Minerva Endocrinologica
EDIZIONI MINERVA MEDICA

OXIDATIVE STRESS AS A KEY FEATURE OF AUTOIMMUNE THYROIDITIS: AN UPDATE.

Journal: Minerva Endocrinologica
Paper code: Minerva Endocrinol-3268
Submission date: June 8, 2020
Article type: Review Article

Files:

1. Reply letter to comments on the manuscript
   Version: 3
   Description: response to referees with highlighted modified text
   File format: application/vnd.openxmlformats-officedocument.wordprocessingml.document

2. Manuscript
   Version: 6
   Description: revised manuscript as suggested by the referees
   File format: application/vnd.openxmlformats-officedocument.wordprocessingml.document

3. Figures 1
   Version: 1
   Description: Figure 1
   File format: image/jpeg

4. Figures 2
   Version: 1
   Description: Figure 2 A) and B)
   File format: image/jpeg

We thank the Editor and the reviewers for the interest in our work and for their constructive comments. We have accepted the suggestions and comments and have modified the manuscript accordingly. To facilitate the reviewing process, the sentences added and/or changed are highlighted in yellow in the text. We provide a point-by-point response to Referees' comments.

Both the Abstract and the Reference list were modified according to journal guidelines, as per Editorial Office's request. The Tables were added at the end of the manuscript file.

We hope that the manuscript is now in good order for publication.

Response to the Editor

We thank the Editor for the interest and the overall favorable evaluation given to our work. We really appreciate his pertinent suggestion to add a short description of the interplay between inflammation, the aging of the thyroid gland (including the anti-oxidative system) and the development of thyroid diseases. The suggested references were quoted.

Response to Referee 1.

We thank referee 1 for the overall favorable evaluation and his/her comments and suggestions, that we carefully met. We modified the manuscript accordingly.

MINOR CORRECTIONS

1. *I suggest authors to discuss about the relationship between HT and thyroid cancer, particularly the papillary histotype. Specifically, authors should report some data and reflect about the interplay between AT, thyroid cancer and oxidative stress. Indeed, an amount of data are available about the relationship between oxidative damage and thyroid carcinogenesis. This may further enrich the paper.*

We thank the Referee for her/his suggestion. We added a paragraph concerning the interplay between autoimmune thyroiditis and thyroid cancer and oxidative stress (in yellow), to further improve the review. Pertinent references were quoted.

2. In the paragraph Possible Therapeutic Role of Antioxidants, I suggest to insert some subparagraphs dedicated to clarify how specific supplements impact on the thyroid oxidative status and the current indications about clinical use. Particularly, authors should refer not only to selenium or LT4 treatment, but also to other possible interventions with antioxidant effect such as VITD supplementation.

Once again, we thank the Referee for this very pertinent comment. We added some subparagraphs to specify the potential role of each specific supplement on the thyroid oxidative status, also discussing the possible antioxidant effects of Vitamin D. Pertinent references were quoted.

3. Please add, if possible basing on journal guidelines, an expert opinion paragraph where authors provide their own considerations and indications about current therapeutic use of antioxidants and suggest strategies of future research and development.

We thank the Referee for his suggestion. We have had our considerations about current and future antioxidant therapeutic strategies in the conclusions.

Responses to Reviewer 2

We thank the Referee for his/her overall favorable and constructive comments.

MINOR CORRECTIONS

The sentence "Therefore, as already demonstrated in vitiligo, also in AT oxidative stress may represent a pathogenetic link between environmental agents and autoimmunity, playing a role both in initiation (novel autoantigens) and progression (autoimmune-related inflammation, cell apoptosis and parenchymal destruction) of the disorder" should be rephrased. In particular, what do the Authors refer to with the term "novel autoantigens". Using this term might lead to confusion, I would suggest to make the sentence more clear.

We thank the Referee for this suggestion. The pertinent sentence was rephrased to make it clearer.

REVISED VERSION OF THE MANUSCRIPT (TEXT ONLY)

Highlighted= added; colored text= modified.

ABSTRACT

Introduction. Oxidative stress has been proposed as one of the factors concurring in the pathophysiology of autoimmune thyroid diseases. Reactive oxygen species are the main expression of oxidative stress in biological systems, and their production can overcome antioxidant defenses ultimately leading to cell damage, apoptosis, and death. The present review was aimed at describing the state of the art of the relationships between oxidative stress and autoimmune thyroiditis. The most used biomarkers of oxidative stress and their correlation with thyroid function are reported.

Evidence Acquisition. We conducted a search of the literature in the English language starting from 2000, using the following search terms: “Hashimoto thyroiditis”, ”autoimmune thyroiditis”, “hypothyroidism”, “hyperthyroidism”, “oxidative stress”, “oxidants”, “antioxidant”, “advanced glycation end products”. Both clinical studies and animal models were evaluated.

Evidence Synthesis. Data form clinical studies clearly indicate that the balance between oxidants and antioxidants is shifted towards the oxidative side in patients with autoimmune thyroiditis, suggesting that oxidative stress may be a key event in the pathophysiology of the disease, irrespective of thyroid function. Studies in animal models, such as the NOD.H2h4 mouse, confirm that thyroidal accumulation of ROS plays a role in the initiation and progression of autoimmune thyroiditis.

Conclusions. Oxidant/antioxidant imbalance represent a key feature of thyroid autoimmunity. Oxidative stress parameters could be used as biochemical markers of chronic inflammation, to better predict the disease evolution along its natural history. Dietary habits and antioxidant supplements may provide protection from autoimmunity, opening new perspectives in the development of more tailored therapies.

INTRODUCTION.

Autoimmune thyroiditis (AT), also referred to as Hashimoto's thyroiditis (HT), is the most common autoimmune endocrine disorder and the main cause of hypothyroidism in iodine-sufficient areas 1,2. AT covers a wide spectrum of phenotypes, encompassing different clinico-pathological entities: the classic form, which features goiter with or without hypothyroidism; the fibrous variant with glandular fibrosis and rapid progression towards hypothyroidism; the IgG4-related variant; the juvenile form and the painless (or silent) thyroiditis, occurring either sporadically or in the post-partum 1,2. Overall, AT has an estimated prevalence of around 3-5% of the general population, with peaks during adolescence and middle adulthood 3, 4. In addition, the prevalence of thyroid autoantibodies increases in people over the age of seventy 5. At any age, females are more affected than males with a female to male ratio around 5-7:1 1,2. Incidence has been raising in last decades, mostly in developed countries 3. AT often occurs in association with other endocrine and non-endocrine autoimmune diseases in the same patient (autoimmune comorbidity) and/or in in other members of the same family (familial clustering), facilitated by a predisposing polygenic background. The clinical presentation of AT can widely vary, from the rapid development of severe hypothyroidism to an initial but transient thyrotoxicosis (Hashitoxicosis). In many cases, AT generally proceeds from an asymptomatic autoimmune condition, featured by circulating anti-thyroid autoantibodies and normal thyroid function, towards subclinical and then overt hypothyroidism 1,2,3,. Even if return to normal thyroid function has been reported, the final outcome of AT is permanent hypothyroidism due to progressive destruction of thyroid follicular cells 1,2.

AT is a chronic inflammation of the thyroid that results from an inappropriate immune reaction against the gland. Failure of immunological self-tolerance leads to activation and expansion of autoreactive T cells, release of pro-inflammatory cytokines and differentiation of self-reactive B cells with production of organ-specific autoantibodies 6. Together, these events are responsible for chronic inflammation with infiltration of hematopoietic mononuclear cells (T and B lymphocytes, plasma cells and macrophages), interstitial fibrosis and follicular cells damage by means of both cell- and antibody-mediated cytotoxic and apoptotic

pathways6. In this cascade of events, a crucial role is played by pro-inflammatory cytokines, such as IL-1β, IL-6, IFN-γ, TNF-α, IL-22 and IL-23, that promote and amplify inflammation, contribute to tissue damage and modulate the metabolic and immune function of thyroid follicular cells (Figure 1) 6,7,8,9.

Underlying this process there is a complex interplay between genetic and environmental factors: exogenous and existential factors trigger the development of the immune response against thyroid autoantigens in genetically susceptible individuals 2,6,10. The genetic background of the disease is still not fully understood and includes a wide number of genes, encompassing the HLA and immune-regulatory genes, that confer generalized susceptibility to autoimmunity on one hand, and several different tissue-specific genes, which exert either predisposing or protective effects for particular types of disease in a tissue-specific fashion on the other 2. In face of the constancy of the genetic basis, the number of potential environmental triggers has been enormously expanding over the last decades. They include changes in lifestyle (modified infectious habitat and ameliorated personal hygiene, stress, dietary habits, sedentary life), increased exposure to pollutants and toxics, radiations, novel (i.e. tyrosine-kinase inhibitors and immune check-point inhibitors) and old (i.e. lithium, interferons, amiodarone) drugs, gut microbiome alterations and nutrients (notably, vitamin D deficiency, iodine and selenium intake) 2,6,10. These environmental factors may affect the thyroid gland and trigger/favor the development of autoimmunity through a wide range of mechanisms, including increased free radicals accumulation and enhanced oxidative stress 11,12.

Oxidative stress is the result of an imbalance between oxidants production and antioxidant defense mechanisms. This condition concerns all the alterations that may occur at tissue, cellular and biological macromolecular level. In biological systems oxidants are represented by free radicals, i.e. partially reduced forms of oxygen and nitrogen, the so-called reactive oxygen (ROS) and nitrogen (RNS) species. From a chemical point of view, a free radical is a molecular entity having one or more unpaired electrons on one atomic or molecular orbital. They present an extremely high reactivity and instability and tend to catch the missing electron from other molecules. Free radicals start chain reactions leading to shutdown of initial radical and/or to the generation of new radicals. These molecules are products of normal cell metabolism and are essential for several biochemical processes inside the cell when at low levels. On the contrary, the alteration of the normal redox state due to their excessive production and/or accumulation into the cells causes oxidation of all macromolecules (membrane lipids, proteins and nucleic acids), leading to cell damage, apoptosis and death 13. Indeed, as postulated in the ‘redox window’ hypothesis, adequate ROS levels help physiological cellular functions, but excessive ROS production is involved in the development of several pathologies 14. The main types of ROS that can be generated in the cell are superoxide (O2•-) anion, hydroxyl (OH•) radical, and hydrogen peroxide (H2O2) which is a non-free radical. There are several enzymes involved in ROS production. At mitochondrial level, the complex I and III are implicated in the production of large amounts of superoxide, and their activity is slowed during increased ROS production which, in turn, promotes further ROS release 15. Moreover, mito-ROS can subsequently activate other ROS sources16. NAD(P)H (nicotinamide adenine dinucleotide phosphate) oxidase catalyzes the release of

superoxide anion or hydrogen peroxide through the reduction of molecular oxygen, using as electron donor NADPH, in various intracellular and extracellular compartments 17. Nitric oxide synthase (NOS) enzyme is responsible for NO production but excessive superoxide levels, in condition of oxidative stress, depletes (6R)5,6,7,8-tetrahydrobiopterin (BH4), the essential NOS cofactor 18, causing NOS impairment, which becomes itself a source of superoxide. Xanthine oxidase (XO), released during pathophysiological processes, donates electrons to molecular oxygen producing superoxide and hydrogen peroxide (Figure 2).

Multiple enzymatic and non-enzymatic systems, the so-called antioxidants, are present in both the bloodstream and peripheral tissues, to prevent/counteract excess free radicals' production and/or accumulation. The enzymatic defenses can remove radicals with a catalytic mechanism, while the non-enzymatic defenses have heterogeneous working mechanisms, as they can sequestrate pro-oxidant molecules, or act as radical scavenger. Non-enzymatic antioxidants may be either endogenous, like the mitochondrial uncoupling proteins, reduced glutathione (GSH) and transport proteins synthetized in the liver (ceruloplasmin, transferrin, albumin etc...) or exogenous products, including uric acid, vitamin E and C, which are mainly derived from diet 13. The enzymatic antioxidants include the glutathione peroxidase (GPx)/glutathione reductase (GR) system and the glutathione-S-transferases (GSTs), which represent the first-line defense against oxidants in almost every cell types, with a tissue-specific distribution (Figure 2). GPx/GR system reduces H2O2 or hydroperoxides reduction, using GSH as electron donor and NADPH as a co-factor, meanwhile GSTs are a class of enzymes which catalyzes the conjugation of GSH to electrophilic compounds 19. Thioredoxin (TRx)/thioredoxin reductase (TRxR) constitutes another important system for H2O2 detoxification. TRxR acts using NADPH to restore the oxidized thioredoxin in the reduced form. Both GPx and TRxR are selenoproteins, with a Se atom incorporated in their catalytic domain in the form of selenocysteine, and require an appropriate supply of Se to be active 20. At the highest levels of oxidants, also catalases (CAT) and superoxide dismutase (SOD) contribute to enzymatic degradation of free radicals 21.

Under physiological conditions, there is an equilibrium between the production and detoxification of free radicals, the so-called redox homeostasis, which is essential for every kind of aerobic life 20,22. When free radicals are produced in excess or are not adequately degraded by the cells or both, then a condition of oxidative stress occurs and causes cell damage and death, and tissue inflammation, also worsened by the release of pro-inflammatory cytokines in damaged tissues 20,22. Almost every cell type and tissue are prone to oxidative damage, with tissue-specific differences, and oxidative stress is present at the site of inflammation23.

For these reasons, oxidative stress has been thought to represent a key feature of several inflammatory and immune-related disorders, including autoimmune thyroid diseases (AITDs) 24,25,26. Increased ROS production due to environmental agents (i.e. iodine excess, radiations, toxics and drugs, pollutants) could induce a modification of tissue proteins, or might dysregulate the immune system, influencing the appearance of the autoimmune disorder 24.

The present review was aimed at summarizing the evidence of the recent literature concerning the bidirectional association between oxidative stress and AT, since the relevance of oxidative stress and the beneficial effects of antioxidant supplementation in ATs are intensely debated at present. We also revised the different biomarkers that have been measured to evaluate the impact of ROS in the setting of AT, in an effort to identify useful and reliable markers of oxidative stress.

EVIDENCE ACQUISITION.

For this purpose, we extensively examined both in vivo and in vitro data on changes in oxidative balance and oxidative stress markers/indices, published in the last two decades in the field of AITDs. The review of the pertinent literature has been conducted employing MEDLINE database. On this website, we searched for articles using key terms related to Hashimoto's thyroiditis and oxidative stress. A MeSH search has been performed using "Hashimoto thyroiditis"/"autoimmune thyroiditis" AND "oxidative stress", followed by a simple search using "Hashimoto thyroiditis", "autoimmune thyroiditis" AND "oxidative stress" OR "oxidants" OR "antioxidant" OR "advanced glycation end products" and a search using "oxidative stress" AND autoimmune thyroiditis" OR "hypothyroidism" OR "hyperthyroidism" as key terms. We obtained 196 results; we included in the present paper only the articles matching the following inclusion criteria: English language, publication in peer-reviewed journals starting from 2000, research papers. Articles considered relevant and cited in the references of the selected papers were included too. We excluded articles for irrelevance to the topic in question, duplicates, papers written in other languages apart from English and articles published before 2000.

EVIDENCE SYNTHESIS.

Animal Models.

The NOD-H2(h4) mouse represents an animal model of autoimmune lymphocytic thyroiditis that mimics human Hashimoto's thyroiditis. The NOD-H2h4 mice spontaneously develop an autoimmune thyroiditis, whose incidence dramatically increases when adding iodine to the drinking water 27,28,29,30. Excess iodine triggers autoimmunity by multiple mechanisms, including changing the immunogenicity of the thyroglobulin molecule, upregulating intracellular adhesion molecule-1 (ICAM-1) expression on thyrocytes and increasing ROS production by the thyrocytes themselves 26. Burek and co-workers demonstrated that thyrocytes isolated from NOD-H2h4 mice produced significantly more H2O2 than control thyrocytes when exposed to iodine26. ROS accumulation also contributes to upregulation of ICAM-1 expression on the surface of thyrocytes, enhancing immune cells infiltration of the thyroid gland and pro-inflammatory cytokines production 31,32. Incubation with the antioxidant diphenyleneiodium, an inhibitor of NADPH oxidase, reduced both ROS production and ICAM-1 expression in cultured NOD-H2h4 thyrocytes 32. Kolypetri and Carayanniotis showed that impaired control of oxidative stress mechanisms is associated with high susceptibility to apoptosis in NOD.H2(h4) thyrocytes exposed to iodine 33 . Finally, the antioxidant N-acetylcysteine (NAC) reduced ROS and the immune infiltration, thereby leading to a restoration of thyroid

morphology, in NOD.H2h4 thyroid glands 34. Likely NAC exert its protective effects by acting on infiltrating inflammatory cells rather than directly on thyrocytes. In the same model, increased thyroid content of 4-HNE, a toxic product from lipid peroxidation used as a marker of oxidative stress, was reported 34. Overall, studies in the NOD.H2h4 model suggest that thyroidal accumulation of ROS plays a role in the initiation and progression of autoimmune thyroiditis.

CLINICAL STUDIES.

Thyroid Function and Oxidative Stress.

There is a close and bidirectional relationship between the thyroid gland and oxidative stress since it concerns both the effects of thyroid function on oxidants/antioxidants balance in peripheral tissues and the effects of oxidative stress on thyroid gland itself.

Firstly, thyroid hormones play a crucial role in regulating redox homeostasis. On the one hand, they accelerate the basal metabolic rate and cell oxidative metabolism by inducing mitochondrial respiration, and enhance free radical production; on the other, they regulate the synthesis of enzymatic and non-enzymatic antioxidants. As a consequence, both hyperthyroidism and hypothyroidism have been associated with oxidative stress, since the former has been shown to increase oxidants production as a result of increase of metabolic processes into cells and to cause consequent exhaustion of antioxidants 35,36,37,38,39,40 and the latter to reduce the antioxidant defense systems 25,37,39,41,42,43 .

Moreover, the thyroid itself is exposed to lifelong oxidative stress, induced by the continuous generation of H2O2, fundamental for the iodine oxidation during the process of thyroid hormone synthesis. For this reason, thyroid cells display efficient detoxification systems, mainly represented by the cytoplasmic GPx1 and by the secretive GPx3 in the colloid lumen 44,45. Inadequate Se supplementation may impair both the expression and the enzymatic activity of the antioxidant selenoproteins GPx1 and GPx3, altering the natural oxidant/antioxidant thyroid cycle and resulting in a reduced antioxidant activity in thyrocytes 21. In animal models, Se deficiency is associated to oxidative cell damage, defective tissue repair and thyroid fibrosis, whilst Se supplementation is protective against experimentally induced autoimmune thyroiditis 46,47. Similarly, in in vitro studies Se supplementation prevents cell damage from oxidative injury and protects against apoptosis 48,49,50. Also, iodine deficiency may increase oxidative stress, by uncoupling H2O2 generation, which is stimulated by TSH in response to iodine deficiency, from iodine oxidation, which is reduced because of iodine deficiency. As a result, excess H2O2 accumulates in the colloid lumen. When combined selenium deficiency occurs, it results in decreased expression of the selenoprotein GPx3 by thyrocytes. As a result, inadequate GPx activity does not remove excess H2O2, which cannot be consumed by thyroperoxidase (TPO) for tyrosine iodination and iodothyronine coupling under conditions of iodine deficiency, leading to H2O2-induced tissue necrosis and fibrosis 21. Hypothyroidism enhances such an effect because H2O2 production, which is increased under continuous and elevated stimulation of the thyroid gland by TSH, is not adequately counteract because of the reduced synthesis of antioxidants, as above

reported 51. A high iodine intake might cause an excessive H2O2 generation too, as clearly demonstrated in animal models 52,32

Finally, oxidative stress seems to have a relevant role in the aging of thyroid gland and in the pathogenesis of age-related thyroid dysfunction. Aging is associated with a decrease in thyroid volume and hormone secretion, as well as with a reset of the hypothalamic–pituitary–thyroid axis 53. The production of free radicals and ROS gradually increases with aging, whereas the activity of antioxidant defenses decreases, leading to ROS accumulation in cells and tissues 54. The oxidative stress caused by this imbalance might contribute to the progressive age-related dysfunction of the thyroid gland directly, through cellular oxidative damage, and indirectly, through alterations in protein synthesis and function. Prolonged age-dependent ROS exposure also causes genomic damage and telomere shortening, contributing to accumulation of senescent cells in the thyroid with age. Moreover, ROS excess favors inflammation by increasing immune cells recruitment into tissues (potentially damaging), by stimulating pro-inflammatory cytokines synthesis/release and by modulating other processes such as mithocondrial function and microRNA production. A chronic, low-level inflammation is a key feature of aging process, the so-called "inflammaging", and a major contributor to both thyroid senescence and age-related thyroid disorders 53,54 . Indeed, aging is associated with an increase in the prevalence of several thyroid diseases, and solid evidence indicates that a condition of oxidative stress is relevant for their development and/or progression.

Thyroid Autoimmunity and Oxidative Stress.

A close relationship exists between oxidative stress and thyroid autoimmunity irrespective of thyroid dysfunction 55,56,57,58. In autoimmune disorders such as AT, the infiltrating immune cells develop a chronic inflammatory milieu in which ROS accumulate and exert a toxic effect on surrounding cells and tissues. In these conditions, oxidative stress may play a role in both the induction of autoimmune response against self-antigens, and the amplification of tissue inflammation and damage, once the autoimmune process has been initiated 24,26. First, oxidative imbalance may play a role in the onset of the autoimmune response. ROS excess causes oxidative modifications of proteins, lipids and DNA, which become highly immunogenic and may act as neo-antigens, leading to loss of self-tolerance in genetically predisposed individuals 56,57. Thyroidal accumulation of ROS has been shown to promote cleavage of thyroglobulin into several fragments, likely exposing the immune system to novel epitopes and thus enhancing the autoimmune response 59. Once the autoimmune reaction has been triggered, the related inflammation may promote excess ROS production and enhanced oxidative stress in thyroid tissue via activation of T and B lymphocytes infiltrating the gland. In fact, it has been demonstrated that Th1 cytokines released by activated lymphocytes induce ROS production by thyrocytes 60,61. Activated lymphocytes themselves produce excess ROS 62,63. Whatever the source is, ROS accumulation causes oxidative damage of the cells, leading to apoptosis, necrosis, and parenchymal destruction, as it occurs in other autoimmune diseases 11,64,65. Moreover, the antioxidant system is not sufficient to counteract ROS overproduction, since the antioxidant potential is reduced in HT patients, even in euthyroidism 25,55,56,57,58. Therefore, as already demonstrated

in vitiligo, also in AT oxidative stress may play a role both in initiation (modified proteins acting as neo-antigens) and progression (autoimmune-related inflammation, cell apoptosis and parenchymal destruction) of the disorder 25,26. Studies in euthyroid patients with AT are more limited than those in hypo- and hyperthyroid patients, but they all agree on demonstrating higher oxidative stress in AT cases than in controls, due to increased oxidants or decreased antioxidants or both 25,55,56,57,58 . In each study a significant correlation with thyroid autoimmunity was found. Ates et al reported a negative correlation between serum total antioxidant activity and anti-thyroperoxidase antibodies (TPO-Ab), while Baser et al. reported a positive correlation between serum oxidants and anti- thyroglobulin (Tg-Ab) antibodies 55, and Ruggeri et al. confirmed the TPO-Ab were independent predictors of the oxidative status in euthyroid HT patients 56. Overall, human studies report an increased oxidative status in AT, even in euthyroidism, but do not clarify whether it is the cause or the consequence of thyroid autoimmunity. Maybe autoimmunity and oxidative stress coexist and act in synergism in initiating and/or perpetrating the progressive damage of thyrocytes.

Autoimmunity, cancer and oxidative stress.

The relationship between AT and thyroid cancer, especially papillary thyroid cancer (PTC), is a well-known fact 66,67 and several research groups have studied the role played by oxidative stress in thyroid carcinogenesis, reporting an increase in levels of oxidants and/or a decrease in antioxidant activity in patients with thyroid cancer 68,69. The accumulation of excess ROS in the thyroid gland can cause DNA damage, resulting in mutagenic genetic alterations and promoting tumour initiation and development 70,71. Thus, it is conceivable that inflammation and oxidative stress, that are closely related processes, may contribute to the increased risk of thyroid cancer that has been reported in AT 67,71, whilst antioxidant may exert protective effects 72.However, scanty data are available concerning the interplay between AT, thyroid cancer and oxidative stress. Lassoued et al evaluated the presence of OS markers in patients suffering from AITDs (Graves' disease and Hashimoto's Thyroiditis) and patients with PTC, before and after thyroidectomy and radioiodine therapy 73. Comparing their oxidative stress profile with that of HT patients, malondialdehyde (MDA), SOD and CAT activities were high, with reduced levels of GPx, in both groups. However, the absolute values were higher (and lower regarding GPx), in the PTC patients, thus suggesting a higher grade of oxidative stress in this population deriving from a more sustained production of free radicals and/or a damaged antioxidant system 73. Moreover, these alterations did not change after thyroidectomy and radioiodine therapy, thus confirming a previous evidence of an intrinsic oxidative imbalance in PTC 74 .Such data could be a starting point to further analyze the potential diagnostic/prognostic role of OS parameters in PTC, also considering the use of antioxidant compounds to ameliorate patients' recovery 73.

Biomarkers of Oxidative Stress.

Hashimoto's thyroiditis (HT)

Starting from 2000s, growing evidence emerged concerning several peripheral/circulating markers of oxidative stress in HT patients (Table 1). In 2006 Taddei et al. demonstrated that patients with HT and subclinical hypothyroidism presented with higher C-reactive protein and IL-6 values. In these subjects, the antioxidant vitamin C did not improve endothelial dysfunction and nitric oxide (NO) availability after the administration of indomethacin, that unselectively blocked a COX2-dependent pathway 75. In 2008 Erdamar et al. observed an increase in MDA, nitrite, vitamin E, and myeloperoxidase (MPO) activity in hypothyroid HT patients, as well as high levels of MDA and MPO activity in hyperthyroid subjects with Graves' disease (GD). Treatments for both conditions revealed a reduction in nitrite and vitamin E in HT patients and a decrease of the raised parameters in GD ones versus a homogenous group of healthy controls. In particular, levothyroxine (L-T4) therapy took two months to lead markers back to normal values, while a faster response (one month) was observed with propylthiouracil (PTU) treatment for hyperthyroidism, thus confirming a role of thyroid hormones oscillations into influencing the redox homeostasis of thyroid gland 37. A decrease in plasmatic levels of transforming growth factor-beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF), and an increase in nitrite/nitrate (NOx, metabolites deriving from NO), was observed in a group of HT patients versus controls 76. In the study by Torun and co-workers, MDA was elevated in both hypothyroid and subclinical hypothyroid patients compared with controls and showed a correlation with altered lipid metabolism in hypothyroidism states. On the contrary, total antioxidant status TAS levels show no significant differences between groups, suggesting an insufficient increase in the antioxidant status in hypothyroid patients 42. In the study by Lassoued et al. oxidative stress in patients with untreated HT and GD resulted higher than in healthy controls, especially concerning SOD activities and MDA. Besides, the same evidence with more elevated values, was observed in patients with surgically treated PTC, thus demonstrating a disturbed oxidative profile as in autoimmune diseases 73. As stated before, oxidative stress could also be influenced by TSH levels, as demonstrated by Ozturk and colleagues who evaluated oxidative stress parameters in a cohort of HT patients, differently affected by subclinical (SHypo) or overt hypothyroidism (OHypo). Several serum parameters (MDA, diene conjugate – DC, protein carbonyl – PC, nitrotyrosine – NT and ferric reducing antioxidant power - FRAP) were altered in comparison with healthy controls, but while MDA and DC levels were normal in SHypo, all these analytes were increased in the OHypo group, and Dc and copper-induced MDA were also measurable in low-density lipoprotein (LDL) fraction in OHypo patients only 77. Moreover, even GSH status has been investigated in HT. As demonstrated by Rostami et al., serum glutathione was significantly reduced in affected subjects versus controls, and it correlated with TPO-Ab values, thus suggesting that this decrease could be a hallmark of oxidative stress activation and immunological intolerance development 78. In the study by Reddy and coworkers, MDA and GPx values were elevated, while GSH, TAC as FRAP, SOD, and SOD/GPx ratio were decreased in hypothyroid HT patient compared to controls, the observed decrease being more relevant in overt than in SHypo HT patients. Thus, hypothyroid subjects displayed deficient antioxidant defenses in relation to the degree of hormonal dysfunction and lipid peroxidation 43. Oxidative stress can act at thyroxisome level, that is impairing the homeostasis of the thyroid hormone-producing unit in the follicle

apical membrane, composed by TPO, Caveolin-1 (Cav-1) and dual oxidase (DUOX). It has been observed that all these components were reduced in HT, in which the Th1 immune response could down-regulate Cav-1 expression, leading to a mislocalization of TPO and DUOX and a decrease of T4 synthesis in the colloid, with consequent oxidative stress and cell apoptosis as main features of HT pathogenesis 61. However, system perturbations involve the whole redox balance, being not only limited to an increased production of reactive species. For example, Ates et al. demonstrated raised total oxidant status (TOS) and oxidative stress index (OSI), as well a reduction in total antioxidant status (TAS), total thiol and ARE levels in HT patients in comparison with healthy volunteers. Indeed, these alterations were progressively more marked passing from euthyroid to subclinical or overt hypothyroid subjects, with a negative correlation between TAS and TPO-Ab 25. The same group showed how TSH, FT4 and OSI ratio could have an independent predictive role of progression from euthyroidism to subclinical, and finally overt, hypothyroidism in HT 79. A similar evidence was found by Ruggeri et al., who analyzed the redox status of a group of euthyroid HT patients in comparison with healthy controls. In the first group, a significant decrease in biological antioxidant potential (BAP), as well as increased levels of derived reactive oxygen metabolites (dROMs) and advanced glycation end products (AGEs), that were both inversely correlated to the former. Moreover, TPO-Ab were the main predictors for all the aforementioned parameters 56. Also, a common polymorphism of AGEs receptor (RAGE) related to HT, namely -429T>C, has been associated with the risk of progression from euthyroidism to hypothyroidism, since patients under L-T4 treatment presented with higher oxidative stress levels 80. AGEs are well known to be increased in conditions of oxidative stress and to promote inflammation by interacting with their receptor RAGE on cell membrane. By contrast, the soluble receptor sRAGE exerts protective effects by competing with RAGE for ligand binding. More recently, reduced levels of the soluble form of RAGE (sRAGE) have been reported in euthyroid HT patients compared to controls, along with increased serum AGEs levels, and the two parameters were inversely correlated 58. Accordingly, the AGEs/sRAGEs ratio was threefold higher in HT patients than controls, suggesting a dysregulation of AGE/sRAGEs-related oxidative homeostasis in HT patients even when in euthyroid status. In regression analysis models, serum TPO-Ab were the main predictors for AGEs and sRAGEs levels and AGEs/sRAGEs ratio, irrespective of TSH and/or FT4 values 58. In other experiences, patients with AITDs presented with reduced levels of paraoxonase-1 (PON1) and total free sulfhydryl (-SH) levels – both compounds having a well-known antioxidant function – while lipid oxidation expressed as lipid hydroperoxide (LOOH) values was significantly higher versus healthy controls 81. The same reduction of PON, and arylesterase (ARE) was observed in a group of female adolescents with euthyroid HT, and it was paired with significantly higher levels of anti-Mullerian hormone 82 . Finally, an increase in serum interleukin-37 (IL-37) has been recently observed in HT patients vs controls, directly correlating with anti-thyroid antibodies titre and AGEs levels. This evidence could lead to hypothesize a protective role of IL-37 against oxidative stress in HT 57.

Graves' disease

Most of the above reported biomarkers have been investigated also in Graves' disease patients, since autoimmune hyperthyroidism and the related orbitopathy are well known to be oxidative stress-related disorders 83. For the sake of completeness and comparison, we briefly report the more recent data on oxidative stress biomarkers in GD patients (Table 2).

As stated before, in 2010 Lassoued et al. demonstrated high levels of SOD activity and MDA in patients with GD 73. Also, metalloprotease (MMP) expression is stimulated by a high-oxidative stress environment, as observed by Korkmaz et al. in a cohort of GD patients without Graves' orbitopathy (GO). They presented high levels of the MMP prolidase, which positively correlated with TOS/OSI indexes, while -SH groups were significantly reduced 84. A reduction in native and total thiol levels in GD patients was also observed by Agan et al, with a positive correlation between free triiodothyronine (FT3) and FT4 levels and thiol homeostasis impairment/oxidative stress parameters 85. High levels of MDA bound to proteins or carbonyl groups, as well as a hyper-reactivity towards hydrogen peroxide (H2O2)-oxidized thyroid antigens, has been observed in patients affected by AITD as expression of oxidative stress presence/increase 86. Indeed, the high production of H2O2 during hormone synthesis could enhance the antigen reactivity through the creation of new epitopes. In GD patients this phenomenon had a positive correlation with FT3 levels 86. Gargouri et al also demonstrated a positive correlation between T3 levels and the immunoreactivity towards MDA-modified catalase in GD patients vs controls 87. In a study by Choi et al, MDA and 8-hydroxy-2'-deoxyquanosine (8-OHdG), H2O2 and intracellular superoxide anion levels were measured in the tear fluid of GD patients 88. These markers resulted increased in comparison to healthy controls, and progressively higher in affected subjects without and with GO, respectively. There was also a positive correlation between markers and clinical activity score (CAS) in GO, while increased levels of extracellular ROS were demonstrated in fibroadipose tissue, blood, orbital fibroblasts, and urine from these subjects 88. Marique et al. detected an increased expression of oxidative stress parameters in both adipose and muscular orbital cells in patients with GO. On the other hand, an upregulation of some antioxidants (peroxiredoxin 5, catalase) was also observed, while the overexpression of adiponectin (ApN) and proliferator-activated receptor gamma (PPARγ) exerted direct and indirect protective effects. These data confirmed the role of antioxidant supplementation in active and chronic GO 89. In another study, increased serum concentrations of nicotinamide adenine dinucleotide phosphate oxidase, isoform 2 (NOX2) were measured in untreated hyperthyroid GD patients, being significantly higher than in GD euthyroid treated patients, subjects with multinodular toxic goiter and healthy controls. Moreover, higher oxidative stress parameters were also detected in urine samples of untreated GD patients, as well as an increased respiratory burst of leukocytes in whole blood 90. In the same study by Diana et al, the Authors measured superoxide production in human embryonic kidney (HEK)-293 cells with overexpressed TSH receptor (TSHR), and lipid peroxidation in these cells and in human primary thyrocytes. Monoclonal M22 TSAbs, bovine TSH and sera from hyperthyroid GD patients stimulated cAMP in HEK cells, significantly increasing superoxide levels vs controls. However, in this case there was no correlation between T3 levels and ROS production. A similar result was obtained in primary thyrocytes, with higher oxidative stress parameters in GD patients vs controls,

and in untreated GD patients vs controls 90. Recently, Ko et al demonstrated a significant increase in ROS production and decrease in antioxidant enzymes in cultured orbital fibroblasts from GD patients with GO, induced by H2O2 or cigarette smoke extract. Of note, treatment with caffeine determined a dose-dependent decrease in intracellular ROS and antioxidant enzymes levels, while PPARγ, C/EBPα, and C/EBPβ protein expression levels were inhibited during adipocyte differentiation 91.

Possible Therapeutic Role of Antioxidants.

Several antioxidant compounds have been evaluated to counteract oxidative stress in thyroid disorders and their role in clinical practice is under debate.

Selenium.

Selenium, a well-known element involved in thyroid homeostasis, has been demonstrated to exert some antioxidant effects in AITDs, although its practical applications are still a matter of debate especially concerning AT. In fact, thyrocytes express many selenoproteins, some of which, like type 1 and 2 iodothyronine deiodinases (DIO1 and 2) and GPX (isoforms 1, 3 and 4), are linked to thyroid hormone metabolism and participate into controlling oxidative stress levels in the gland 92. Others, like selenoprotein S (SELENOS), modulate the transcription of genes encoding proinflammatory cytokines involved in AT pathogenesis 93. Selenium intake is widely variable in the world due to environmental reasons and dietary habits, ranging from deficiency to toxicity doses (7-4990 μg/day), and in Europe it is in general under the levels recommended by the US Institute of Medicine or the European Food Safety Authority (EFSA), that is 55 and 70 μg/day respectively 94.

In vitro studies demonstrated that incubation of thyrocytes with Se supplements is able to prevent oxidative cell necrosis and apoptosis and enhances cell viability by preventing H2O2-induced degradation of DNA and by reducing caspase-3 activity and BAX mRNA levels and increasing BCL-2 mRNA levels 46–50. An increase in MDA levels, which was prevented by the pretreatment with both selenomethionine and selenite, was also reported after exposure to H2O2 50. Both selenocompounds induced an increase in GPx activity, suggesting that these protective effects may be, almost in part, mediated by these selenoproteins 50.

A number of studies evaluating selenium supplementation in HT have demonstrated a reduction in TPO-Ab concentration, but there are scarce evidences about its effects on disease progression and remission, influences on L-T4 replacement dosage and patients' quality of life 95,94. For example, the addition of selenomethionine 200 μg/day to levothyroxine treatment in a cohort of 170 Polish women with HT vs 41 controls demonstrated a reduced release of proinflammatory cytokines comprehending IL-2, IFNγ and TNF, and decreased levels of C-reactive protein 96. On the converse, another Austrian study in women with HT treated with levothyroxine plus sodium selenite 200 μg/day for three months did not record significant modifications in cytokine profile 97. While some ongoing trials are evaluating this association, the paradoxical aspect to be clarified is that, in a recent survey performed by the European Thyroid Association

(ETA), around 65% of European endocrinologists occasionally or frequently prescribe selenium despite its use in HT is not routinely recommended 94,98.

The use of selenium in mild and active GO is an established tool in clinical practice, but the same consensus has not been extended to the treatment of Graves' disease without GO 40,99,100. In fact, even if one study investigating the effects of selenium added to methimazole (MMI) treatment for GD reported a good biochemical control of hyperthyroidism, two randomized clinical trials (RCTs) did not demonstrated a short-term improvement of hyperthyroidism or an amelioration of response/recurrence rates 101,102,103. However, new evidences could emerge from more recent studies thoroughly analysing clinical parameters, like the work by Xu et al, in which the comparison between MMI alone and MMI plus selenium groups revealed significantly lower levels of FT3, FT4, TPO-Ab, Tg-Ab and TRAb, and a marked increase in TSH after 6 months of treatment 104.

Vitamin antioxidants.

Vitamin antioxidant compounds, such as vitamin C, vitamin E, β-carotene, have been employed in Graves' disease patients under MMI or carbimazole therapy with positive results, providing evidence in favour of the use of these antioxidant supplements in the early phases of anti-thyroid treatment 105. For example, Guerra et al tested a combination between antithyroid drugs and an antioxidant mixture (vitamin E, β-carotene, vitamin C, Cu, Zn, Mn, and selenium) vs anti-thyroid drugs alone in hyperthyroid GD patients: in the first group, a significant reduction in MDA levels was recorded, with a shorter time required to normalize thyroid hormones and the clinical score, thus suggesting a synergistic mechanism at thyroid hormone synthesis level 106. Larger cohort of patients are needed to confirm these findings.

In recent years, a growing importance of the abovementioned vitamin antioxidants, namely alpha-tocopherol and ascorbate, has been linked to a better efficiency of the antioxidant systems and the endogenous antioxidants like SOD and CAT, as observed for example in benign prostate hyperplasia (BPH), in which ROS production could contribute to carcinogenesis 107. A similar evidence has been observed in a group of thyroid cancer patients, treated with radioiodine and supplemented with 2000 mg vitamin C, 1000 mg vitamin E, and 400 µg selenium for 21 days before therapy. They presented with significantly lower plasma levels of 8-epi-PGF2a, a marker of lipid peroxidation, thus allowing to extend the hypothesis of a beneficial effects of these substances in AITDs 108.

Vitamin D.

Vitamin D is a steroid hormone, which has been demonstrated to be involved in several processes in the human body, including the regulation of immune response. In fact, its receptor (vitamin D receptor, VDR) is present in monocytes and activated T cells, influencing both the innate and adaptive immunity 109,110. Several observational studies showed a relationship between vitamin D deficiency and the risk of developing AITDs, especially HT, and a strong negative correlation has been observed between reduced vitamin D

levels and TPO-Ab, whereas the association of HT with VDR polymorphisms was variable 111–113. Moreover, this connection could also be confirmed by the fact that, conversely, vitamin D can downregulate the expression of proinflammatory cytokines, such as Interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), which are responsible for T and B cells migration and proliferation and for an increase in oxidative stress levels 114. In the majority of randomized controlled trials (6 out of 7) conducted up to now in HT patients, the main effect of cholecalciferol supplementation is a significant reduction of TPO-Ab titer, but several questions remain unanswered i.e. regarding the best dosage, the best detection method, etc 110. Considering these data, an empirical approach could be to consider measuring vitamin D levels in patients affected by HT, especially when they are middle aged-women (the most affected part of population) and/or presenting with slightly elevated TSH levels: this passage could increase the number of patients treated with vitamin D, not only for the prevention of bone metabolism alterations, but also in order to slow down the progression of HT towards overt hypothyroidism and/or thyroid atrophy 115. Similar considerations could be applied in GD patients, but further studies with larger cohorts of patients and more stringent selection criteria are required.

Dietary intake of plant-derived antioxidants.

Plant-based foods are rich sources of vitamins, oligoelements and metabolites (such as phenols, polyphenols, phytoestrogens, flavonoids, flavones, proanthocyanins, catechins) with documented antioxidant activity and may exert protective effects against oxidative stress-related disorders, including autoimmune processes. In isolated rural populations a vegetarian diet has been associated with a low prevalence of autoimmune diseases, such a protective influence being attributed to the effects of plant foods, to the exclusion of animal products, or both. On the contrary, a Western-type diet, rich in calories, fats, and proteins, high in salt and refined sugars, and low in fibers, is thought to favor the development of autoimmunity through enhanced oxidative stress and inflammation. Indeed, consumption of large amounts of meat, fats and refined sugar in the long run results in gut microbiota disruption and inflammation with ROS overproduction, while the low intake of fruits and vegetables causes lack of exogenous antioxidants 116. In the last decade, Tonstad et al., using data from the Adventist Health Study-2, reported a reduced prevalence and incidence of both hypothyroidism and hyperthyroidism among subjects following vegan/vegetarian diets compared to omnivorous diets, providing congruent, though not always statistically significant, data in favour of a protective role of diet excluding meat against thyroid dysfunction 117,118. More recently, Ruggeri and co-workers provided evidence that low dietary intake of animal foods has a potentially protective effect towards thyroid autoimmunity as a result of the positive influence of this dietary habit on redox balance119. According to this survey, the nutritional pattern of HT subjects was characterized by increased consumption of animal proteins, higher intake of saturated fats and refined sugars, and lower intake of fibers and antioxidants compared with healthy control subjects. In other words, nutritional patterns of HT subjects resembled the Western-type diet, while controls displayed a higher level of adherence to the Mediterranean diet. The study points to meat in omnivorous diets as the main nutritional factor associated

with redox dysregulation and increased risk of thyroid autoimmunity, whilst plant-based foods and Mediterranean diet traits are protective119.

L-T4 treatment.

Another interesting topic is the role of thyroid hormone replacement therapy towards oxidative stress, with many evidences emerging in the literature. In a study by Marchiori et al, in a cohort of 17 hypothyroid HT patients treated with L-T4, oxidative stress parameters were measured at 6 and 12 months, respectively. A significant reduction in non-protein thiol (NP-SH) was observed, together with significant modifications in interleukin levels (increase in IL-10 and decreases in IL-1, IL-6, INF-γ and TNF-α), thus suggesting that hormone replacement therapy could condition the inflammatory response in thyroid autoimmunity 120. A similar effectiveness of L-T4 was reported in a paper by Ates et al, in which a group of treated HT patients presented with increased levels of TAS, total thiol, ARE, and PON1 and decreased TOS and OSI levels in a 6-month period, compared to healthy controls. Moreover, pre-treatment TOS and OSI levels positively correlated with TSH values, TPO- and Tg-Ab titer 121. More recently, another group recorded an increase in antioxidant CAT levels, and a significant decrease in thiobarbituric acid reactive substances (TBARS) in a group of 25 female patients with primary hypothyroidism treated with L-T4 122. On the other hand, Chakrabarti et al. described the effects of the association of L-T4 replacement therapy with selenium supplementation (100 mcg twice a day) in hypothyroid HT patients, vs L-T4 alone: although in both groups a reduction in MDA levels was recorded after 6 months, there was no statistical significance in the measurements obtained in the combination group 123.

CONCLUSION.

In the context of a growing amount of data about the role of oxidative stress and antioxidants in the pathophysiology of AITDs, and especially in AT progression towards hypothyroidism, oxidative stress parameters could be used as biochemical markers of chronic inflammation, to better predict the disease evolution along its natural history. Besides, the well-known link between oxidative alterations and thyroid hormone replacement treatment could be the theoretical basis to use these parameters for a finer patients' monitoring. A larger knowledge of the substances which locally counteract the oxidative stress imbalance typical of chronic inflammatory disorders could open new perspectives in the development of more tailored medical therapies for these autoimmune conditions that significantly impair patients' quality of life. Currently, natural sources of antioxidants in the form of a plant-based foods may represent the best option for protecting against chronic oxidative stress-related disorders. Reducing the intake of animal proteins and fats and increasing that of fruits and vegetables has proven to be a useful lifestyle strategy for contrasting oxidative stress and reducing the risk for autoimmune diseases, including AT. In particular, a predominantly plant-based Mediterranean diet, high in naturally occurring antioxidants, low in saturated fat and cholesterol, and good source of vitamins and minerals, may represent a healthy food model for people suffering from AT. Conversely, there is still a debate as to whether assuming antioxidants in supplement form can actually

reduce the risk of AT development/progression, by preventing or slowing down thyroid damage. In spite of the experimental evidence for a protective effect of antioxidant molecules against oxidative damage to cells in animal models and in vitro studies, clinical studies of antioxidant supplements in AT patients reached disappointing and largely inconclusive results and have not demonstrate them to provide substantial benefits in preventing/slowing down AT development and progression towards thyroid dysfunction. On this basis, a routine use of vitamins and antioxidants supplementation in the treatment of AT patients should be discouraged, but correction of nutrient deficiencies (for instance, vitamin D or selenium deficit) is advisable to avoid negative health effects due to the lack of these elements essential to proper thyroid and immune function. Moreover, antioxidant supplements cannot be routinely given for a long enough time to prevent chronic diseases, such as HT and hypothyroidism, which develop over decades, but short courses of antioxidants supplementation can be considered as an option and justified in selected conditions and/or populations. A 6-month trial of selenium supplements has indication in the treatment of patients with Graves' disease and associated mild orbitopathy since selenium may enhance the effectiveness of anti-thyroid drugs, improves clinical manifestations and quality of life and prevents progression of the disease. Importantly, current guidelines and scientific societies do not recommend Se supplementation for other indications. Future antioxidant therapeutic strategies should include the design of protocols for the inhibition of oxidative stress damage through administration of synthetic and natural antioxidants and enhancement of the antioxidant defenses by increasing the production of endogenous antioxidants and activation of antioxidant mechanisms.

OXIDATIVE STRESS AS A KEY FEATURE OF AUTOIMMUNE THYROIDITIS: AN UPDATE.

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Running title: oxidative stress and autoimmune thyroiditis

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Key words: Hashimoto's thyroiditis, oxidative stress, ROS, antioxidants, thyroid autoimmunity.

ABSTRACT

Introduction. Oxidative stress has been proposed as one of the factors concurring in the pathophysiology of autoimmune thyroid diseases. Reactive oxygen species are the main expression of oxidative stress in biological systems, and their production can overcome antioxidant defenses ultimately leading to cell damage, apoptosis, and death. The present review was aimed at describing the state of the art of the relationships between oxidative stress and autoimmune thyroiditis. The most used biomarkers of oxidative stress and their correlation with thyroid function are reported.

Evidence Acquisition. We conducted a search of the literature in the English language starting from 2000, using the following search terms: “Hashimoto thyroiditis”, ”autoimmune thyroiditis”, “hypothyroidism”, “hyperthyroidism”, “oxidative stress”, “oxidants”, “antioxidant”, “advanced glycation end products”. Both clinical studies and animal models were evaluated.

Evidence Synthesis. Data form clinical studies clearly indicate that the balance between oxidants and antioxidants is shifted towards the oxidative side in patients with autoimmune thyroiditis, suggesting that oxidative stress may be a key event in the pathophysiology of the disease, irrespective of thyroid function. Studies in animal models, such as the NOD.H2h4 mouse, confirm that thyroidal accumulation of ROS plays a role in the initiation and progression of autoimmune thyroiditis.

Conclusions. Oxidant/antioxidant imbalance represent a key feature of thyroid autoimmunity. Oxidative stress parameters could be used as biochemical markers of chronic inflammation, to better predict the disease evolution along its natural history. Dietary habits and antioxidant supplements may provide protection from autoimmunity, opening new perspectives in the development of more tailored therapies.

INTRODUCTION.

Autoimmune thyroiditis (AT), also referred to as Hashimoto's thyroiditis (HT), is the most common autoimmune endocrine disorder and the main cause of hypothyroidism in iodine-sufficient areas [1,2.] AT covers a wide spectrum of phenotypes, encompassing different clinico-pathological entities: the classic form, which features goiter with or without hypothyroidism; the fibrous variant with glandular fibrosis and rapid progression towards hypothyroidism; the IgG4-related variant; the juvenile form and the painless (or silent) thyroiditis, occurring either sporadically or in the post-partum [1,2]. Overall, AT has an estimated prevalence of around 3-5% of the general population, with peaks during adolescence and middle adulthood [3, 4]. In addition, the prevalence of thyroid autoantibodies increases in people over the age of seventy [5]. At any age, females are more affected than males with a female to male ratio around 5-7:1 [1,2]. Incidence has been raising in last decades, mostly in developed countries [3]. AT often occurs in association with other endocrine and non-endocrine autoimmune diseases in the same patient (autoimmune comorbidity) and/or in in other members of the same family (familial clustering), facilitated by a predisposing polygenic background. The clinical presentation of AT can widely vary, from the rapid development of severe hypothyroidism to an initial but transient thyrotoxicosis (Hashitoxicosis). In many cases, AT generally proceeds from an asymptomatic autoimmune condition, featured by circulating anti-thyroid autoantibodies and normal thyroid function, towards subclinical and then overt hypothyroidism [1,2,3,.] Even if return to normal thyroid function has been reported, the final outcome of AT is permanent hypothyroidism due to progressive destruction of thyroid follicular cells [1,2].

AT is a chronic inflammation of the thyroid that results from an inappropriate immune reaction against the gland. Failure of immunological self-tolerance leads to activation and expansion of autoreactive T cells, release of pro-inflammatory cytokines and differentiation of self-reactive B cells with production of organ-specific autoantibodies [6.] Together, these events are responsible for chronic inflammation with infiltration of hematopoietic mononuclear cells (T and B lymphocytes, plasma cells and macrophages), interstitial fibrosis and follicular cells damage by means of both cell- and antibody-mediated cytotoxic and apoptotic pathways[6.] In this cascade of events, a crucial role is played by pro-inflammatory cytokines, such as IL-1β, IL-6, IFN-γ, TNF-α, IL-22 and IL-23, that promote and amplify inflammation, contribute to tissue damage and modulate the metabolic and immune function of thyroid follicular cells (**Figure 1**) [6,7,8,9.]

Underlying this process there is a complex interplay between genetic and environmental factors: exogenous and existential factors trigger the development of the immune response against thyroid autoantigens in genetically susceptible individuals [2,6,10.] The genetic background of the disease is still not fully understood and includes a wide number of genes, encompassing the HLA and immune-regulatory genes, that confer generalized susceptibility to autoimmunity on one hand, and several different tissue-specific genes, which exert either predisposing or protective effects for particular types of disease in a tissue-specific fashion on the other [2]. In face of the constancy of the genetic basis, the number of potential environmental triggers has been enormously expanding over the last decades. They include changes in lifestyle (modified infectious habitat and ameliorated personal hygiene, stress, dietary habits, sedentary life), increased exposure to pollutants and toxics, radiations, novel (i.e. tyrosine-kinase inhibitors and immune check-point inhibitors) and old (i.e. lithium, interferons, amiodarone) drugs, gut microbiome alterations and nutrients (notably, vitamin D deficiency, iodine and selenium intake) [2,6,10.] These environmental factors may affect the thyroid gland and trigger/favor the development of autoimmunity through a wide range of mechanisms, including increased free radicals accumulation and enhanced oxidative stress [11,12].

Oxidative stress is the result of an imbalance between oxidants production and antioxidant defense mechanisms. This condition concerns all the alterations that may occur at tissue, cellular and biological macromolecular level. In biological systems oxidants are represented by free radicals, i.e. partially reduced forms of oxygen and nitrogen, the so-called reactive oxygen (ROS) and nitrogen (RNS) species. From a chemical point of view, a free radical is a molecular entity having one or more unpaired electrons on one atomic or molecular orbital. They present an extremely high reactivity and instability and tend to catch the missing electron from other molecules. Free radicals start chain reactions leading to shutdown of initial radical and/or to the generation of new radicals. These molecules are products of normal cell metabolism and are essential for several biochemical processes inside the cell when at low levels. On the contrary, the alteration of the normal redox state due to their excessive production and/or accumulation into the cells causes oxidation of all macromolecules (membrane lipids, proteins and nucleic acids), leading to cell damage, apoptosis and death [13]. Indeed, as postulated in the 'redox window' hypothesis, adequate ROS levels help physiological cellular functions, but excessive ROS production is involved in the development of several pathologies [14]. The main types of ROS that can be generated in the cell are superoxide (O2•-) anion, hydroxyl (OH•) radical, and hydrogen peroxide (H_2O_2) which is a non-free radical. There are several

enzymes involved in ROS production. At mitochondrial level, the complex I and III are implicated in the production of large amounts of superoxide, and their activity is slowed during increased ROS production which, in turn, promotes further ROS release [15]. Moreover, mito-ROS can subsequently activate other ROS sources[16]. NAD(P)H (nicotinamide adenine dinucleotide phosphate) oxidase catalyzes the release of superoxide anion or hydrogen peroxide through the reduction of molecular oxygen, using as electron donor NADPH, in various intracellular and extracellular compartments [17]. Nitric oxide synthase (NOS) enzyme is responsible for NO production but excessive superoxide levels, in condition of oxidative stress, depletes (6R)5,6,7,8-tetrahydrobiopterin (BH4), the essential NOS cofactor [18], causing NOS impairment, which becomes itself a source of superoxide. Xanthine oxidase (XO), released during pathophysiological processes, donates electrons to molecular oxygen producing superoxide and hydrogen peroxide (**Figure 2**).

Multiple enzymatic and non-enzymatic systems, the so-called antioxidants, are present in both the bloodstream and peripheral tissues, to prevent/counteract excess free radicals' production and/or accumulation. The enzymatic defenses can remove radicals with a catalytic mechanism, while the non-enzymatic defenses have heterogeneous working mechanisms, as they can sequestrate pro-oxidant molecules, or act as radical scavenger. Non-enzymatic antioxidants may be either endogenous, like the mitochondrial uncoupling proteins, reduced glutathione (GSH) and transport proteins synthetized in the liver (ceruloplasmin, transferrin, albumin etc..) or exogenous products, including uric acid, vitamin E and C, which are mainly derived from diet [13]. The enzymatic antioxidants include the glutathione peroxidase (GPx)/glutathione reductase (GR) system and the glutathione-S-transferases (GSTs), which represent the first-line defense against oxidants in almost every cell types, with a tissue-specific distribution (**Figure 2**). GPx/GR system reduces H_2O_2 or hydroperoxides reduction, using GSH as electron donor and NADPH as a co-factor, meanwhile GSTs are a class of enzymes which catalyzes the conjugation of GSH to electrophilic compounds [19]. Thioredoxin (TRx)/thioredoxin reductase (TRxR) constitutes another important system for H2O2 detoxification. TRxR acts using NADPH to restore the oxidized thioredoxin in the reduced form. Both GPx and TRxR are selenoproteins, with a Se atom incorporated in their catalytic domain in the form of selenocysteine, and require an appropriate supply of Se to be active [20]. At the highest levels of oxidants, also catalases (CAT) and superoxide dismutase (SOD) contribute to enzymatic degradation of free radicals [21].

Under physiological conditions, there is an equilibrium between the production and detoxification of free radicals, the so-called redox homeostasis, which is essential for every kind of aerobic life [20,22]. When free

radicals are produced in excess or are not adequately degraded by the cells or both, then a condition of oxidative stress occurs and causes cell damage and death, and tissue inflammation, also worsened by the release of pro-inflammatory cytokines in damaged tissues [20,22]. Almost every cell type and tissue are prone to oxidative damage, with tissue-specific differences, and oxidative stress is present at the site of inflammation[23].

For these reasons, oxidative stress has been thought to represent a key feature of several inflammatory and immune-related disorders, including autoimmune thyroid diseases (AITDs) [24,25,26]. Increased ROS production due to environmental agents (i.e. iodine excess, radiations, toxics and drugs, pollutants) could induce a modification of tissue proteins, or might dysregulate the immune system, influencing the appearance of the autoimmune disorder [24].

The present review was aimed at summarizing the evidence of the recent literature concerning the bidirectional association between oxidative stress and AT, since the relevance of oxidative stress and the beneficial effects of antioxidant supplementation in ATs are intensely debated at present. We also revised the different biomarkers that have been measured to evaluate the impact of ROS in the setting of AT, in an effort to identify useful and reliable markers of oxidative stress.

EVIDENCE ACQUISITION.

For this purpose, we extensively examined both *in vivo* and *in vitro* data on changes in oxidative balance and oxidative stress markers/indices, published in the last two decades in the field of AITDs. The review of the pertinent literature has been conducted employing MEDLINE database. On this website, we searched for articles using key terms related to Hashimoto's thyroiditis and oxidative stress. A MeSH search has been performed using "Hashimoto thyroiditis"/"autoimmune thyroiditis" AND "oxidative stress", followed by a simple search using "Hashimoto thyroiditis", "autoimmune thyroiditis" AND "oxidative stress" OR "oxidants" OR "antioxidant" OR "advanced glycation end products" and a search using "oxidative stress" AND autoimmune thyroiditis" OR "hypothyroidism" OR "hyperthyroidism" as key terms. We obtained 196 results; we included in the present paper only the articles matching the following inclusion criteria: English language, publication in peer-reviewed journals starting from 2000, research papers. Articles considered relevant and cited in the references of the selected papers were included too. We excluded articles for irrelevance to the topic in question, duplicates, papers written in other languages apart from English and articles published before 2000.

EVIDENCE SYNTHESIS.

Animal Models.

The NOD-H2(h4) mouse represents an animal model of autoimmune lymphocytic thyroiditis that mimics human Hashimoto's thyroiditis. The NOD-H2h4 mice spontaneously develop an autoimmune thyroiditis, whose incidence dramatically increases when adding iodine to the drinking water [27,28,29,30.] Excess iodine triggers autoimmunity by multiple mechanisms, including changing the immunogenicity of the thyroglobulin molecule, upregulating intracellular adhesion molecule-1 (ICAM-1) expression on thyrocytes and increasing ROS production by the thyrocytes themselves [26]. Burek and co-workers demonstrated that thyrocytes isolated from NOD-H2h4 mice produced significantly more H_2O_2 than control thyrocytes when exposed to iodine[26]. ROS accumulation also contributes to upregulation of ICAM-1 expression on the surface of thyrocytes, enhancing immune cells infiltration of the thyroid gland and pro-inflammatory cytokines production [31],[32]. Incubation with the antioxidant diphenyleneiodium, an inhibitor of NADPH oxidase, reduced both ROS production and ICAM-1 expression in cultured NOD-H2h4 thyrocytes [32]. Kolypetri and Carayanniotis showed that impaired control of oxidative stress mechanisms is associated with high susceptibility to apoptosis in NOD.H2(h4) thyrocytes exposed to iodine [33] . Finally, the antioxidant N-acetylcysteine (NAC) reduced ROS and the immune infiltration, thereby leading to a restoration of thyroid morphology, in NOD.H2h4 thyroid glands [34]. Likely NAC exert its protective effects by acting on infiltrating inflammatory cells rather than directly on thyrocytes. In the same model, increased thyroid content of 4-HNE, a toxic product from lipid peroxidation used as a marker of oxidative stress, was reported [34]. Overall, studies in the NOD.H2h4 model suggest that thyroidal accumulation of ROS plays a role in the initiation and progression of autoimmune thyroiditis.

CLINICAL STUDIES.

Thyroid Function and Oxidative Stress.

There is a close and bidirectional relationship between the thyroid gland and oxidative stress since it concerns both the effects of thyroid function on oxidants/antioxidants balance in peripheral tissues and the effects of oxidative stress on thyroid gland itself.

Firstly, thyroid hormones play a crucial role in regulating redox homeostasis. On the one hand, they accelerate the basal metabolic rate and cell oxidative metabolism by inducing mitochondrial respiration, and enhance free radical production; on the other, they regulate the synthesis of enzymatic and non-enzymatic

antioxidants. As a consequence, both hyperthyroidism and hypothyroidism have been associated with oxidative stress, since the former has been shown to increase oxidants production as a result of increase of metabolic processes into cells and to cause consequent exhaustion of antioxidants [35,36,37,38,39,40] and the latter to reduce the antioxidant defense systems [25,37,39,41,42,43] .

Moreover, the thyroid itself is exposed to lifelong oxidative stress, induced by the continuous generation of H_2O_2, fundamental for the iodine oxidation during the process of thyroid hormone synthesis. For this reason, thyroid cells display efficient detoxification systems, mainly represented by the cytoplasmic GPx1 and by the secretive GPx3 in the colloid lumen [44,45.] Inadequate Se supplementation may impair both the expression and the enzymatic activity of the antioxidant selenoproteins GPx1 and GPx3, altering the natural oxidant/antioxidant thyroid cycle and resulting in a reduced antioxidant activity in thyrocytes [21]. In animal models, Se deficiency is associated to oxidative cell damage, defective tissue repair and thyroid fibrosis, whilst Se supplementation is protective against experimentally induced autoimmune thyroiditis [46,47]. Similarly, in *in vitro* studies Se supplementation prevents cell damage from oxidative injury and protects against apoptosis [48,49,50]. Also, iodine deficiency may increase oxidative stress, by uncoupling H_2O_2 generation, which is stimulated by TSH in response to iodine deficiency, from iodine oxidation, which is reduced because of iodine deficiency. As a result, excess H_2O_2 accumulates in the colloid lumen. When combined selenium deficiency occurs, it results in decreased expression of the selenoprotein GPx3 by thyrocytes. As a result, inadequate GPx activity does not remove excess H_2O_2, which cannot be consumed by thyroperoxidase (TPO) for tyrosine iodination and iodothyronine coupling under conditions of iodine deficiency, leading to H_2O_2-induced tissue necrosis and fibrosis [21]. Hypothyroidism enhances such an effect because H_2O_2 production, which is increased under continuous and elevated stimulation of the thyroid gland by TSH, is not adequately counteract because of the reduced synthesis of antioxidants, as above reported [51]. A high iodine intake might cause an excessive H_2O_2 generation too, as clearly demonstrated in animal models [52,32]

Finally, oxidative stress seems to have a relevant role in the aging of thyroid gland and in the pathogenesis of age-related thyroid dysfunction. Aging is associated with a decrease in thyroid volume and hormone secretion, as well as with a reset of the hypothalamic–pituitary–thyroid axis [53]. The production of free radicals and ROS gradually increases with aging, whereas the activity of antioxidant defenses decreases, leading to ROS accumulation in cells and tissues [54]. The oxidative stress caused by this imbalance might

contribute to the progressive age-related dysfunction of the thyroid gland directly, through cellular oxidative damage, and indirectly, through alterations in protein synthesis and function. Prolonged age-dependent ROS exposure also causes genomic damage and telomere shortening, contributing to accumulation of senescent cells in the thyroid with age. Moreover, ROS excess favors inflammation by increasing immune cells recruitment into tissues (potentially damaging), by stimulating pro-inflammatory cytokines synthesis/release and by modulating other processes such as mithocondrial function and microRNA production. A chronic, low-level inflammation is a key feature of aging process, the so-called "inflammaging", and a major contributor to both thyroid senescence and age-related thyroid disorders [53,54] . Indeed, aging is associated with an increase in the prevalence of several thyroid diseases, and solid evidence indicates that a condition of oxidative stress is relevant for their development and/or progression.

Thyroid Autoimmunity and Oxidative Stress.

A close relationship exists between oxidative stress and thyroid autoimmunity irrespective of thyroid dysfunction [55,56,57,58]. In autoimmune disorders such as AT, the infiltrating immune cells develop a chronic inflammatory milieu in which ROS accumulate and exert a toxic effect on surrounding cells and tissues. In these conditions, oxidative stress may play a role in both the induction of autoimmune response against self-antigens, and the amplification of tissue inflammation and damage, once the autoimmune process has been initiated [24,26]. First, oxidative imbalance may play a role in the onset of the autoimmune response. ROS excess causes oxidative modifications of proteins, lipids and DNA, which become highly immunogenic and may act as neo-antigens, leading to loss of self-tolerance in genetically predisposed individuals [56,57]. Thyroidal accumulation of ROS has been shown to promote cleavage of thyroglobulin into several fragments, likely exposing the immune system to novel epitopes and thus enhancing the autoimmune response [59]. Once the autoimmune reaction has been triggered, the related inflammation may promote excess ROS production and enhanced oxidative stress in thyroid tissue via activation of T and B lymphocytes infiltrating the gland. In fact, it has been demonstrated that Th1 cytokines released by activated lymphocytes induce ROS production by thyrocytes [60,61]. Activated lymphocytes themselves produce excess ROS [62,63]. Whatever the source is, ROS accumulation causes oxidative damage of the cells, leading to apoptosis, necrosis, and parenchymal destruction, as it occurs in other autoimmune diseases [11,64,65]. Moreover, the antioxidant system is not sufficient to counteract ROS overproduction, since the antioxidant potential is reduced in HT patients, even in euthyroidism [25,55,56,57,58]. Therefore, as already demonstrated in vitiligo, also

in AT oxidative stress may play a role both in initiation (modified proteins acting as neo-antigens) and progression (autoimmune-related inflammation, cell apoptosis and parenchymal destruction) of the disorder [25,26]. Studies in euthyroid patients with AT are more limited than those in hypo- and hyperthyroid patients, but they all agree on demonstrating higher oxidative stress in AT cases than in controls, due to increased oxidants or decreased antioxidants or both [25,55,56,57,58] . In each study a significant correlation with thyroid autoimmunity was found. Ates et al reported a negative correlation between serum total antioxidant activity and anti-thyroperoxidase antibodies (TPO-Ab), while Baser et al. reported a positive correlation between serum oxidants and anti- thyroglobulin (Tg-Ab) antibodies [55], and Ruggeri et al. confirmed the TPO-Ab were independent predictors of the oxidative status in euthyroid HT patients [56]. Overall, human studies report an increased oxidative status in AT, even in euthyroidism, but do not clarify whether it is the cause or the consequence of thyroid autoimmunity. Maybe autoimmunity and oxidative stress coexist and act in synergism in initiating and/or perpetrating the progressive damage of thyrocytes.

Autoimmunity, cancer and oxidative stress.

The relationship between AT and thyroid cancer, especially papillary thyroid cancer (PTC), is a well-known fact [66,67] and several research groups have studied the role played by oxidative stress in thyroid carcinogenesis, reporting an increase in levels of oxidants and/or a decrease in antioxidant activity in patients with thyroid cancer [68,69]. The accumulation of excess ROS in the thyroid gland can cause DNA damage, resulting in mutagenic genetic alterations and promoting tumour initiation and development [70,71]. Thus, it is conceivable that inflammation and oxidative stress, that are closely related processes, may contribute to the increased risk of thyroid cancer that has been reported in AT [67,71], whilst antioxidant may exert protective effects [72].However, scanty data are available concerning the interplay between AT, thyroid cancer and oxidative stress. Lassoued et al evaluated the presence of OS markers in patients suffering from AITDs (Graves' disease and Hashimoto's Thyroiditis) and patients with PTC, before and after thyroidectomy and radioiodine therapy [73]. Comparing their oxidative stress profile with that of HT patients, malondialdehyde (MDA), SOD and CAT activities were high, with reduced levels of GPx, in both groups. However, the absolute values were higher (and lower regarding GPx), in the PTC patients, thus suggesting a higher grade of oxidative stress in this population deriving from a more sustained production of free radicals and/or a damaged antioxidant system [73]. Moreover, these alterations did not change after thyroidectomy and radioiodine therapy, thus confirming a previous evidence of an intrinsic oxidative imbalance in PTC [74] .Such

data could be a starting point to further analyze the potential diagnostic/prognostic role of OS parameters in PTC, also considering the use of antioxidant compounds to ameliorate patients' recovery [73].

***Biomarkers of Oxidative Stress*.**

Hashimoto's thyroiditis (HT)

Starting from 2000s, growing evidence emerged concerning several peripheral/circulating markers of oxidative stress in HT patients (**Table 1**). In 2006 Taddei et al. demonstrated that patients with HT and subclinical hypothyroidism presented with higher C-reactive protein and IL-6 values. In these subjects, the antioxidant vitamin C did not improve endothelial dysfunction and nitric oxide (NO) availability after the administration of indomethacin, that unselectively blocked a COX2-dependent pathway [75]. In 2008 Erdamar et al. observed an increase in MDA, nitrite, vitamin E, and myeloperoxidase (MPO) activity in hypothyroid HT patients, as well as high levels of MDA and MPO activity in hyperthyroid subjects with Graves' disease (GD). Treatments for both conditions revealed a reduction in nitrite and vitamin E in HT patients and a decrease of the raised parameters in GD ones *versus* a homogenous group of healthy controls. In particular, levothyroxine (L-T4) therapy took two months to lead markers back to normal values, while a faster response (one month) was observed with propylthiouracil (PTU) treatment for hyperthyroidism, thus confirming a role of thyroid hormones oscillations into influencing the redox homeostasis of thyroid gland [37]. A decrease in plasmatic levels of transforming growth factor-beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF), and an increase in nitrite/nitrate (NOx, metabolites deriving from NO), was observed in a group of HT patients versus controls [76]. In the study by Torun and co-workers, MDA was elevated in both hypothyroid and subclinical hypothyroid patients compared with controls and showed a correlation with altered lipid metabolism in hypothyroidism states. On the contrary, total antioxidant status TAS levels show no significant differences between groups, suggesting an insufficient increase in the antioxidant status in hypothyroid patients [42]. In the study by Lassoued et al. oxidative stress in patients with untreated HT and GD resulted higher than in healthy controls, especially concerning SOD activities and MDA. Besides, the same evidence with more elevated values, was observed in patients with surgically treated PTC, thus demonstrating a disturbed oxidative profile as in autoimmune diseases [73]. As stated before, oxidative stress could also be influenced by TSH levels, as demonstrated by Ozturk and colleagues who evaluated oxidative stress parameters in a cohort of HT patients, differently affected by subclinical (SHypo) or overt hypothyroidism (OHypo). Several serum parameters (MDA, diene conjugate – DC, protein carbonyl – PC,

nitrotyrosine – NT and ferric reducing antioxidant power - FRAP) were altered in comparison with healthy controls, but while MDA and DC levels were normal in SHypo, all these analytes were increased in the OHypo group, and Dc and copper-induced MDA were also measurable in low-density lipoprotein (LDL) fraction in OHypo patients only [77]. Moreover, even GSH status has been investigated in HT. As demonstrated by Rostami et al., serum glutathione was significantly reduced in affected subjects *versus* controls, and it correlated with TPO-Ab values, thus suggesting that this decrease could be a hallmark of oxidative stress activation and immunological intolerance development [78]. In the study by Reddy and coworkers, MDA and GPx values were elevated, while GSH, TAC as FRAP, SOD, and SOD/GPx ratio were decreased in hypothyroid HT patient compared to controls, the observed decrease being more relevant in overt than in SHypo HT patients. Thus, hypothyroid subjects displayed deficient antioxidant defenses in relation to the degree of hormonal dysfunction and lipid peroxidation [43]. Oxidative stress can act at thyroxisome level, that is impairing the homeostasis of the thyroid hormone-producing unit in the follicle apical membrane, composed by TPO, Caveolin-1 (Cav-1) and dual oxidase (DUOX). It has been observed that all these components were reduced in HT, in which the Th1 immune response could down-regulate Cav-1 expression, leading to a mislocalization of TPO and DUOX and a decrease of T4 synthesis in the colloid, with consequent oxidative stress and cell apoptosis as main features of HT pathogenesis [61]. However, system perturbations involve the whole redox balance, being not only limited to an increased production of reactive species. For example, Ates et al. demonstrated raised total oxidant status (TOS) and oxidative stress index (OSI), as well a reduction in total antioxidant status (TAS), total thiol and ARE levels in HT patients in comparison with healthy volunteers. Indeed, these alterations were progressively more marked passing from euthyroid to subclinical or overt hypothyroid subjects, with a negative correlation between TAS and TPO-Ab [25]. The same group showed how TSH, FT4 and OSI ratio could have an independent predictive role of progression from euthyroidism to subclinical, and finally overt, hypothyroidism in HT [79]. A similar evidence was found by Ruggeri et al., who analyzed the redox status of a group of euthyroid HT patients in comparison with healthy controls. In the first group, a significant decrease in biological antioxidant potential (BAP), as well as increased levels of derived reactive oxygen metabolites (dROMs) and advanced glycation end products (AGEs), that were both inversely correlated to the former. Moreover, TPO-Ab were the main predictors for all the aforementioned parameters [56]. Also, a common polymorphism of AGEs receptor (RAGE) related to HT, namely -429T>C, has been associated with the risk of progression from euthyroidism

to hypothyroidism, since patients under L-T4 treatment presented with higher oxidative stress levels [80]. AGEs are well known to be increased in conditions of oxidative stress and to promote inflammation by interacting with their receptor RAGE on cell membrane. By contrast, the soluble receptor sRAGE exerts protective effects by competing with RAGE for ligand binding. More recently, reduced levels of the soluble form of RAGE (sRAGE) have been reported in euthyroid HT patients compared to controls, along with increased serum AGEs levels, and the two parameters were inversely correlated [58]. Accordingly, the AGEs/sRAGEs ratio was threefold higher in HT patients than controls, suggesting a dysregulation of AGE/sRAGEs-related oxidative homeostasis in HT patients even when in euthyroid status. In regression analysis models, serum TPO-Ab were the main predictors for AGEs and sRAGEs levels and AGEs/sRAGEs ratio, irrespective of TSH and/or FT4 values [58]. In other experiences, patients with AITDs presented with reduced levels of paraoxonase-1 (PON1) and total free sulfhydryl (-SH) levels – both compounds having a well-known antioxidant function – while lipid oxidation expressed as lipid hydroperoxide (LOOH) values was significantly higher *versus* healthy controls [81]. The same reduction of PON, and arylesterase (ARE) was observed in a group of female adolescents with euthyroid HT, and it was paired with significantly higher levels of anti-Mullerian hormone [82] . Finally, an increase in serum interleukin-37 (IL-37) has been recently observed in HT patients *vs* controls, directly correlating with anti-thyroid antibodies titre and AGEs levels. This evidence could lead to hypothesize a protective role of IL-37 against oxidative stress in HT [57].

Graves' disease

Most of the above reported biomarkers have been investigated also in Graves' disease patients, since autoimmune hyperthyroidism and the related orbitopathy are well known to be oxidative stress-related disorders [83]. For the sake of completeness and comparison, we briefly report the more recent data on oxidative stress biomarkers in GD patients (**Table 2**).

As stated before, in 2010 Lassoued et al. demonstrated high levels of SOD activity and MDA in patients with GD [73]. Also, metalloprotease (MMP) expression is stimulated by a high-oxidative stress environment, as observed by Korkmaz et al. in a cohort of GD patients without Graves' orbitopathy (GO). They presented high levels of the MMP prolidase, which positively correlated with TOS/OSI indexes, while -SH groups were significantly reduced [84]. A reduction in native and total thiol levels in GD patients was also observed by Agan et al, with a positive correlation between free triiodothyronine (FT3) and FT4 levels and thiol homeostasis impairment/oxidative stress parameters [85]. High levels of MDA bound to proteins or carbonyl

groups, as well as a hyper-reactivity towards hydrogen peroxide (H_2O_2)-oxidized thyroid antigens, has been observed in patients affected by AITD as expression of oxidative stress presence/increase [86]. Indeed, the high production of H_2O_2 during hormone synthesis could enhance the antigen reactivity through the creation of new epitopes. In GD patients this phenomenon had a positive correlation with FT3 levels [86]. Gargouri et al also demonstrated a positive correlation between T3 levels and the immunoreactivity towards MDA-modified catalase in GD patients *vs* controls [87]. In a study by Choi et al, MDA and 8-hydroxy-2'-deoxyquanosine (8-OHdG), H_2O_2 and intracellular superoxide anion levels were measured in the tear fluid of GD patients [88]. These markers resulted increased in comparison to healthy controls, and progressively higher in affected subjects without and with GO, respectively. There was also a positive correlation between markers and clinical activity score (CAS) in GO, while increased levels of extracellular ROS were demonstrated in fibroadipose tissue, blood, orbital fibroblasts, and urine from these subjects [88]. Marique et al. detected an increased expression of oxidative stress parameters in both adipose and muscular orbital cells in patients with GO. On the other hand, an upregulation of some antioxidants (peroxiredoxin 5, catalase) was also observed, while the overexpression of adiponectin (ApN) and proliferator-activated receptor gamma (PPARγ) exerted direct and indirect protective effects. These data confirmed the role of antioxidant supplementation in active and chronic GO [89]. In another study, increased serum concentrations of nicotinamide adenine dinucleotide phosphate oxidase, isoform 2 (NOX2) were measured in untreated hyperthyroid GD patients, being significantly higher than in GD euthyroid treated patients, subjects with multinodular toxic goiter and healthy controls. Moreover, higher oxidative stress parameters were also detected in urine samples of untreated GD patients, as well as an increased respiratory burst of leukocytes in whole blood [90]. In the same study by Diana et al, the Authors measured superoxide production in human embryonic kidney (HEK)-293 cells with overexpressed TSH receptor (TSHR), and lipid peroxidation in these cells and in human primary thyrocytes. Monoclonal M22 TSAbs, bovine TSH and sera from hyperthyroid GD patients stimulated cAMP in HEK cells, significantly increasing superoxide levels vs controls. However, in this case there was no correlation between T3 levels and ROS production. A similar result was obtained in primary thyrocytes, with higher oxidative stress parameters in GD patients vs controls, and in untreated GD patients vs controls [90]. Recently, Ko et al demonstrated a significant increase in ROS production and decrease in antioxidant enzymes in cultured orbital fibroblasts from GD patients with GO, induced by H_2O_2 or cigarette smoke extract. Of note, treatment with caffeine determined a dose-dependent

decrease in intracellular ROS and antioxidant enzymes levels, while PPARγ, C/EBPα, and C/EBPβ protein expression levels were inhibited during adipocyte differentiation [91].

Possible Therapeutic Role of Antioxidants.

Several antioxidant compounds have been evaluated to counteract oxidative stress in thyroid disorders and their role in clinical practice is under debate.

Selenium.

Selenium, a well-known element involved in thyroid homeostasis, has been demonstrated to exert some antioxidant effects in AITDs, although its practical applications are still a matter of debate especially concerning AT. In fact, thyrocytes express many selenoproteins, some of which, like type 1 and 2 iodothyronine deiodinases (DIO1 and 2) and GPX (isoforms 1, 3 and 4), are linked to thyroid hormone metabolism and participate into controlling oxidative stress levels in the gland [92]. Others, like selenoprotein S (SELENOS), modulate the transcription of genes encoding proinflammatory cytokines involved in AT pathogenesis [93]. Selenium intake is widely variable in the world due to environmental reasons and dietary habits, ranging from deficiency to toxicity doses (7-4990 μg/day), and in Europe it is in general under the levels recommended by the US Institute of Medicine or the European Food Safety Authority (EFSA), that is 55 and 70 μg/day respectively [94].

In vitro studies demonstrated that incubation of thyrocytes with Se supplements is able to prevent oxidative cell necrosis and apoptosis and enhances cell viability by preventing H_2O_2-induced degradation of DNA and by reducing caspase-3 activity and BAX mRNA levels and increasing BCL-2 mRNA levels [46–50]. An increase in MDA levels, which was prevented by the pretreatment with both selenomethionine and selenite, was also reported after exposure to H_2O_2 [50]. Both selenocompounds induced an increase in GPx activity, suggesting that these protective effects may be, almost in part, mediated by these selenoproteins [50].

A number of studies evaluating selenium supplementation in HT have demonstrated a reduction in TPO-Ab concentration, but there are scarce evidences about its effects on disease progression and remission, influences on L-T4 replacement dosage and patients' quality of life [95,94]. For example, the addition of selenomethionine 200 μg/day to levothyroxine treatment in a cohort of 170 Polish women with HT *vs* 41 controls demonstrated a reduced release of proinflammatory cytokines comprehending IL-2, IFNγ and TNF, and decreased levels of C-reactive protein [96]. On the converse, another Austrian study in women with HT treated with levothyroxine plus sodium selenite 200 μg/day for three months did not record significant

modifications in cytokine profile [97]. While some ongoing trials are evaluating this association, the paradoxical aspect to be clarified is that, in a recent survey performed by the European Thyroid Association (ETA), around 65% of European endocrinologists occasionally or frequently prescribe selenium despite its use in HT is not routinely recommended [94,98].

The use of selenium in mild and active GO is an established tool in clinical practice, but the same consensus has not been extended to the treatment of Graves' disease without GO [40,99,100.] In fact, even if one study investigating the effects of selenium added to methimazole (MMI) treatment for GD reported a good biochemical control of hyperthyroidism, two randomized clinical trials (RCTs) did not demonstrated a short-term improvement of hyperthyroidism or an amelioration of response/recurrence rates [101,102,103]. However, new evidences could emerge from more recent studies thoroughly analysing clinical parameters, like the work by Xu et al, in which the comparison between MMI alone and MMI plus selenium groups revealed significantly lower levels of FT3, FT4, TPO-Ab, Tg-Ab and TRAb, and a marked increase in TSH after 6 months of treatment [104].

Vitamin antioxidants.

Vitamin antioxidant compounds, such as vitamin C, vitamin E, β-carotene, have been employed in Graves' disease patients under MMI or carbimazole therapy with positive results, providing evidence in favour of the use of these antioxidant supplements in the early phases of anti-thyroid treatment [105]. For example, Guerra et al tested a combination between antithyroid drugs and an antioxidant mixture (vitamin E, β-carotene, vitamin C, Cu, Zn, Mn, and selenium) *vs* anti-thyroid drugs alone in hyperthyroid GD patients: in the first group, a significant reduction in MDA levels was recorded, with a shorter time required to normalize thyroid hormones and the clinical score, thus suggesting a synergistic mechanism at thyroid hormone synthesis level [106]. Larger cohort of patients are needed to confirm these findings.

In recent years, a growing importance of the abovementioned vitamin antioxidants, namely alpha-tocopherol and ascorbate, has been linked to a better efficiency of the antioxidant systems and the endogenous antioxidants like SOD and CAT, as observed for example in benign prostate hyperplasia (BPH), in which ROS production could contribute to carcinogenesis [107]. A similar evidence has been observed in a group of thyroid cancer patients, treated with radioiodine and supplemented with 2000 mg vitamin C, 1000 mg vitamin E, and 400 μg selenium for 21 days before therapy. They presented with significantly lower plasma

levels of 8-epi-PGF2a, a marker of lipid peroxidation, thus allowing to extend the hypothesis of a beneficial effects of these substances in AITDs [108].

Vitamin D.

Vitamin D is a steroid hormone, which has been demonstrated to be involved in several processes in the human body, including the regulation of immune response. In fact, its receptor (vitamin D receptor, VDR) is present in monocytes and activated T cells, influencing both the innate and adaptive immunity [109,110]. Several observational studies showed a relationship between vitamin D deficiency and the risk of developing AITDs, especially HT, and a strong negative correlation has been observed between reduced vitamin D levels and TPO-Ab, whereas the association of HT with VDR polymorphisms was variable [111–113]. Moreover, this connection could also be confirmed by the fact that, conversely, vitamin D can downregulate the expression of proinflammatory cytokines, such as Interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), which are responsible for T and B cells migration and proliferation and for an increase in oxidative stress levels [114]. In the majority of randomized controlled trials (6 out of 7) conducted up to now in HT patients, the main effect of cholecalciferol supplementation is a significant reduction of TPO-Ab titer, but several questions remain unanswered i.e. regarding the best dosage, the best detection method, etc [110]. Considering these data, an empirical approach could be to consider measuring vitamin D levels in patients affected by HT, especially when they are middle aged-women (the most affected part of population) and/or presenting with slightly elevated TSH levels: this passage could increase the number of patients treated with vitamin D, not only for the prevention of bone metabolism alterations, but also in order to slow down the progression of HT towards overt hypothyroidism and/or thyroid atrophy [115]. Similar considerations could be applied in GD patients, but further studies with larger cohorts of patients and more stringent selection criteria are required.

Dietary intake of plant-derived antioxidants.

Plant-based foods are rich sources of vitamins, oligoelements and metabolites (such as phenols, polyphenols, phytoestrogens, flavonoids, flavones, proanthocyanins, catechins) with documented antioxidant activity and may exert protective effects against oxidative stress-related disorders, including autoimmune processes. In isolated rural populations a vegetarian diet has been associated with a low prevalence of autoimmune diseases, such a protective influence being attributed to the effects of plant foods, to the exclusion of animal products, or both. On the contrary, a Western-type diet, rich in calories, fats, and proteins, high in salt and refined sugars, and low in fibers, is thought to favor the development of

autoimmunity through enhanced oxidative stress and inflammation. Indeed, consumption of large amounts of meat, fats and refined sugar in the long run results in gut microbiota disruption and inflammation with ROS overproduction, while the low intake of fruits and vegetables causes lack of exogenous antioxidants [116]. In the last decade, Tonstad et al., using data from the Adventist Health Study-2, reported a reduced prevalence and incidence of both hypothyroidism and hyperthyroidism among subjects following vegan/vegetarian diets compared to omnivorous diets, providing congruent, though not always statistically significant, data in favour of a protective role of diet excluding meat against thyroid dysfunction [117,118]. More recently, Ruggeri and co-workers provided evidence that low dietary intake of animal foods has a potentially protective effect towards thyroid autoimmunity as a result of the positive influence of this dietary habit on redox balance[119]. According to this survey, the nutritional pattern of HT subjects was characterized by increased consumption of animal proteins, higher intake of saturated fats and refined sugars, and lower intake of fibers and antioxidants compared with healthy control subjects. In other words, nutritional patterns of HT subjects resembled the Western-type diet, while controls displayed a higher level of adherence to the Mediterranean diet. The study points to meat in omnivorous diets as the main nutritional factor associated with redox dysregulation and increased risk of thyroid autoimmunity, whilst plant-based foods and Mediterranean diet traits are protective[119].

L-T4 treatment.

Another interesting topic is the role of thyroid hormone replacement therapy towards oxidative stress, with many evidences emerging in the literature. In a study by Marchiori et al, in a cohort of 17 hypothyroid HT patients treated with L-T4, oxidative stress parameters were measured at 6 and 12 months, respectively. A significant reduction in non-protein thiol (NP-SH) was observed, together with significant modifications in interleukin levels (increase in IL-10 and decreases in IL-1, IL-6, INF-γ and TNF-α), thus suggesting that hormone replacement therapy could condition the inflammatory response in thyroid autoimmunity [120]. A similar effectiveness of L-T4 was reported in a paper by Ates et al, in which a group of treated HT patients presented with increased levels of TAS, total thiol, ARE, and PON1 and decreased TOS and OSI levels in a 6-month period, compared to healthy controls. Moreover, pre-treatment TOS and OSI levels positively correlated with TSH values, TPO- and Tg-Ab titer [121]. More recently, another group recorded an increase in antioxidant CAT levels, and a significant decrease in thiobarbituric acid reactive substances (TBARS) in a

group of 25 female patients with primary hypothyroidism treated with L-T4 [122]. On the other hand, Chakrabarti et al. described the effects of the association of L-T4 replacement therapy with selenium supplementation (100 mcg twice a day) in hypothyroid HT patients, *vs* L-T4 alone: although in both groups a reduction in MDA levels was recorded after 6 months, there was no statistical significance in the measurements obtained in the combination group [123].

CONCLUSION.

In the context of a growing amount of data about the role of oxidative stress and antioxidants in the pathophysiology of AITDs, and especially in AT progression towards hypothyroidism, oxidative stress parameters could be used as biochemical markers of chronic inflammation, to better predict the disease evolution along its natural history. Besides, the well-known link between oxidative alterations and thyroid hormone replacement treatment could be the theoretical basis to use these parameters for a finer patients' monitoring. A larger knowledge of the substances which locally counteract the oxidative stress imbalance typical of chronic inflammatory disorders could open new perspectives in the development of more tailored medical therapies for these autoimmune conditions that significantly impair patients' quality of life. Currently, natural sources of antioxidants in the form of a plant-based foods may represent the best option for protecting against chronic oxidative stress-related disorders. Reducing the intake of animal proteins and fats and increasing that of fruits and vegetables has proven to be a useful lifestyle strategy for contrasting oxidative stress and reducing the risk for autoimmune diseases, including AT. In particular, a predominantly plant-based Mediterranean diet, high in naturally occurring antioxidants, low in saturated fat and cholesterol, and good source of vitamins and minerals, may represent a healthy food model for people suffering from AT. Conversely, there is still a debate as to whether assuming antioxidants in supplement form can actually reduce the risk of AT development/progression, by preventing or slowing down thyroid damage. In spite of the experimental evidence for a protective effect of antioxidant molecules against oxidative damage to cells in animal models and *in vitro* studies, clinical studies of antioxidant supplements in AT patients reached disappointing and largely inconclusive results and have not demonstrate them to provide substantial benefits in preventing/slowing down AT development and progression towards thyroid dysfunction. On this basis, a routine use of vitamins and antioxidants supplementation in the treatment of AT patients should be discouraged, but correction of nutrient deficiencies (for instance, vitamin D or selenium deficit) is advisable

to avoid negative health effects due to the lack of these elements essential to proper thyroid and immune function. Moreover, antioxidant supplements cannot be routinely given for a long enough time to prevent chronic diseases, such as HT and hypothyroidism, which develop over decades, but short courses of antioxidants supplementation can be considered as an option and justified in selected conditions and/or populations. A 6-month trial of selenium supplements has indication in the treatment of patients with Graves' disease and associated mild orbitopathy since selenium may enhance the effectiveness of anti-thyroid drugs, improves clinical manifestations and quality of life and prevents progression of the disease. Importantly, current guidelines and scientific societies do not recommend Se supplementation for other indications. Future antioxidant therapeutic strategies should include the design of protocols for the inhibition of oxidative stress damage through administration of synthetic and natural antioxidants and enhancement of the antioxidant defenses by increasing the production of endogenous antioxidants and activation of antioxidant mechanisms.

ACKNOWLEDGEMENT AND DISCLOSURES

Conflict of interests: none of the authors has any conflict of interests.

Funding: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contribution: each Author equally contributed to this paper. All Authors read and approved the final version of the manuscript.

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LEGEND FOR FIGURES.

Figure 1. Schematic representation of ROS involvement in the pathogenesis of autoimmune thyroiditis, in the context of an inflammatory *milieu*. When not counterbalanced by antioxidant mechanisms, excess ROS trigger thyrocytes damage and the release of pro-inflammatory mediators, such as TNF-alpha and IL-1, which in turn activate macrophages and promote the recruitment of immune cells (Th17, Th1 and CD8 cells and neutrophils) via Il-23, IL-17, IL-22 and INF-alpha. The consequent intervention of pro-inflammatory mediators sustains thyroid damage in a cyclic loop.

Figure 2. A) This simplified scheme highlights the connections between the main ROS produced at cellular level. They can lead to several damages like the oxidation of lipids, cytosol/nuclear proteins and nucleic acids, up to the generation of novel autoantigens exacerbating autoimmunity. B) The main actions of catalase and glutathione peroxidase.

ROS: reactive oxygen species; e-: electron; pr+: proton; Fe: iron; SOD: superoxide dismutase; MPO: myeloperoxidase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; NADPH nicotinamide adenine dinucleotide phosphate.

LIST OF ABBREVIATIONS

4-HNE: 4-hydroxynonenal
8-OHdG: 8-hydroxy-2′-deoxyquanosine
AGEs: advanced glycation end products
AMH: anti-Mullerian hormone
Antiox-cap: non-enzymatic antioxidants
AOPP: advanced oxidation protein products
ApN: adiponectin
ARE: arylesterase
ARS: enzymatic antioxidants
AT: autoimmune thyroiditis
BAP: biological antioxidant potential
CAT: catalase activity
C/EBPα and C/EBPβ: CCAAT/enhancer-binding protein alpha and beta
CRP: C-reactive protein
DC: diene conjugate
d-ROMs: derived reactive oxygen metabolites
FRAP: ferric reducing antioxidant power
FT4: free thyroxine
FT3: free triiodothyronine
GD: Graves' disease
GO: Graves' ophthalmopathy
GPx: glutathione peroxidase
GR: glutathione reductase
GSH: Glutathione
IMA: ischemia-modified albumin
HT: Hashimoto's thyroiditis
131I: 131 iodine
IL-6: interleukin 6
IL-37: interleukin 37
L-T4: levothyroxine
LOOH: lipid hydroperoxide
MDA: malondialdehyde
MMI: methimazole
MNTG: multinodular toxic goiter
MPO: myeloperoxidase
NO: nitric oxide
NOX2: Nicotinamide adenine dinucleotide phosphate oxidase, isoform 2;
NT: nitrotyrosine
oLAb: anti-oxidized low-density lipoprotein (LDL) antibodies
ox-LDL: oxidized-low density lipoprotein
OHypo: overt hypothyroidism
OS: oxidative stress
OSI: oxidative stress index
PC: protein carbonyl
PON1: paraoxonase 1
PPARγ: proliferator-activated receptor gamma
RAGE: advanced glycation end products receptor
SH: thiol groups;
SHypo: subclinical hypothyroidism;
SOD: superoxide dismutase;
sRAGE: soluble advanced glycation end products receptor;
TAC: total antioxidant capacity
TGF-β1: transforming growth factor-beta 1
T3: triiodothyronine
TAS: total antioxidant status

Tg-Ab: anti-thyroglobulin antibodies
TOS: total oxidative stress
TOS*: total oxidant status
TPO-Ab: anti-peroxidase antibodies
TRAP: total reactive antioxidant potential
VEGF: vascular endothelial growth factor

TABLES

Table 1. An overview of the studies evaluating the relationship between thyroid autoimmunity, functional status and oxidative stress parameters in patients affected by autoimmune thyroiditis.

Study (Ref.)	Year	Patients	Controls	Thyroid functional status	Oxidative stress indexes/parameters	Results
Ates et al (25)	2015	31 31 31	31	Euthyroidism SHypo OHypo	oxidants: ARE, OSI, SH, TOS antioxidants: PON1, TAS	antioxidants reduction progressively higher towards OHypo
Erdamar et al (37)	2008	20	20	OHypo HT	oxidants: MDA, MPO and nitrites	specific treatment revealed an amelioration in OS
Resch et al (39)	2002	34	34	OHypo	oxidants: oLAb, peroxides antioxidants: Antiox-cap, ARS	enhanced OS; higher oLAb in hypothyroid (atherosclerosis progression index)
Baskol et al (41)	2007	33	26	OHypo (18 euthyroid under therapy)	oxidants: MDA, NO antioxidants: PON1, SOD	significant pro-oxidative imbalance in hypothyroidism, lipid peroxidation linked to atherosclerosis
Torun et al (42)	2009	20 40	40	OHypo SHypo	oxidants: LOOH, MDA antioxidants: TAS other parameters: CRP	pro-oxidative imbalance in both conditions paired with altered lipid metabolism
Reddy et al (43)	2013	36 36	39	OHypo SHypo	oxidants: CAT, LOOH, MDA antioxidants: FRAP, GPx, GR, GSH, SOD, TAC	antioxidants deficiency linked to hypothyroidism severity, higher in overt forms
Baser et al (53)	2014	35	35	Euthyroidism	oxidants: IMA, ox-LDL, TOS* antioxidants: TAS	oxidative stress increased in HT, TAS negatively correlated with thyroid autoantibodies

Ruggeri et al (54)	2016	71	63	Euthyroidism	oxidants: AGEs, AOPP, d-ROMs antioxidants: BAP	TPO-Ab+ related imbalance favoring oxidants
Ruggeri et al (55)	2019	45	50	Euthyroidism	oxidants: AGEs, d-ROMS antioxidants: BAP other parameters: IL-37	Increased oxidative stress; increase in IL-37 as a potential protective factor
Ruggeri et al (56)	2020	50	50	Euthyroidism	oxidants: AGEs antioxidants: sRAGE	high AGEs and low sRAGE levels both correlated with TPO-Ab levels
Taddei et al (64)	2006	53	45	SHypo	oxidants: NO cytokines: IL-6 other parameters: CRP	increased OS markers, no effects of vitamin C on endothelium
Vural et al (65)	2009	40	40	Euthyroidism	oxidants: NO other parameters: TGF-β1, VEGF	increased OS
Lassoued et al (66)	2010	29	30	Untreated OHypo	oxidants: CAT, MDA antioxidants: GPx, SOD	high OS in untreated diseases
Öztürk et al (67)	2012	18 30	30	OHypo SHypo	oxidants: DC, MDA, NT, PC antioxidants: FRAP	increased OS, higher in overt forms
Rostami et al (68)	2013	44	58	Hypo	antioxidants: GSH	decrease of antioxidants correlated with TPO-Ab
Ates et al (69)	2018	40 40	-	Euthyroidism SHypo	oxidants: ARE, OSI, PON1, TOS	TSO independent predictor of progression towards OHypo
Giannakou et al (70)	2017	96 109	95	Treated HT Untreated euthyroid HT	oxidants: RAGE	RAGE polymorphism linked to progression towards OHypo
Korkmaz et al (71)	2016	25	27	Euthyroidism under L-T4 therapy	oxidants: ARE, LOOH, PON1, SH	redox imbalance favoring oxidant compounds
Erol et al (72)	2016	57	50	Euthyroidism	oxidants: AGEs, ARE antioxidants: PON1 other parameters: AMH	antioxidants reduction paired with higher AMH in female adolescents

Korkmaz et al (74)	2015	25	27	Euthyroidism under L-T4 therapy	oxidants: OSI, prolidase, SH, TOS antioxidants: TAS	increase in prolidase correlated with TOS and OSI
Mseddi et al (76)	2017	43	65	SHypo and OHypo HT	oxidants: MDA, SH	high levels of MDA and high immunoreactivity towards oxidized thyroid antigens

Abbreviations: AMH: anti-Mullerian hormone; CRP: C-reactive protein; FT4: free thyroxine; HT: Hashimoto's thyroiditis; IL-6: interleukin 6; IL-37: interleukin 37; L-T4: levothyroxine; OHypo: overt hypothyroidism; OS: oxidative stress; SHypo: subclinical hypothyroidism; T3: triiodothyronine; TPO-Ab: anti-peroxidase antibodies.

OS indexes: BAP: biological antioxidant potential; FRAP: ferric reducing antioxidant power; OSI: oxidative stress index; TAC: total antioxidant capacity; TAS: total antioxidant status; TOS: total oxidative stress; TOS*: total oxidant status; TRAP: total reactive antioxidant potential.

OS biomarkers: AGEs: advanced glycation end products; Antiox-cap: non-enzymatic antioxidants; AOPP: advanced oxidation protein products; ARE: arylesterase; CAT: catalase activity; DC: diene conjugate; d-ROMs: derived reactive oxygen metabolites; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: Glutathione; IMA: ischemia-modified albumin; LOOH: lipid hydroperoxide; MDA: malondialdehyde; MPO: myeloperoxidase; NO: nitric oxide; NT: nitrotyrosine; oLAb: anti-oxidized low-density lipoprotein (LDL) antibodies; ox-LDL: oxidized-low density lipoprotein; PC: protein carbonyl; PON1: paraoxonase 1; RAGE: advanced glycation end products receptor; SH: thiol groups; SOD: superoxide dismutase; sRAGE: soluble advanced glycation end products receptor; TGF-β1: transforming growth factor-beta 1; VEGF: vascular endothelial growth factor.

Table 2. An overview of the studies evaluating the relationship between thyroid autoimmunity, functional status and oxidative stress parameters in patients affected by Graves' disease.

Study (Ref.)	Year	Patients	Controls	Thyroid functional status	Oxidative stress indexes/parameters	Results
Abalovich et al (35)	2003	69	19	Hyperthyroidism	oxidants: CAT, hydroperoxide antioxidants: GPx, GSH, SOD, TRAP	all oxidants reduced after MMI; hydroperoxide remained high after 131I
Bednarek et al (36)	2005	47	24	Hyperthyroidism (22 with GO)	oxidants: CAT, hydroperoxide, ceruloplasmin, LOOH, thiobarbituric acid-reacting substances antioxidants: GPx, GR	MMI reduced markers in patients without GO
Aslan et al (38)	2011	36	30	Hyperthyroidism (21 GD, 15 MNTG)	oxidants: OSI, TOS* antioxidants: TAC	TAC significantly lower, TOS and OSI significantly higher in hyperthyroid patients
Resch et al (39)	2002	34	34	Hyperthyroidism	oxidants: oLAb, peroxides antioxidants: Antiox-cap, ARS	enhanced OS; higher POX in hyperthyroid (hypermetabolic state)
Lassoued et al (66)	2010	16	30	Untreated hyperthyroidism	oxidants: CAT, MDA antioxidants: GPx, SOD	high OS in untreated disease
Korkmaz et al (71)	2016	25	27	Euthyroid treated GD	oxidants: ARE, LOOH, PON1, SH	redox imbalance favoring oxidant compounds
Korkmaz et al (74)	2015	25	27	Euthyroid treated GD	oxidants: prolidase, OSI, SH, TOS antioxidants: TAS	increase in prolidase correlated with TOS and OSI
Agan et al (75)	2019	33	35	Untreated hyperthyroidism	oxidants: OSI, PC, SH, TOS	positive correlation between free thyroid hormones and thiol homeostasis

Mseddi et al (76)	2017	34	65	Untreated hyperthyroidism	oxidants: MDA, SH	high levels of MDA and high immunoreactivity towards oxidized thyroid antigens
Gargouri et al (77)	2019	34	65	Untreated hyperthyroidism	oxidants: CAT	positive correlation between T3 and immunoreactivity towards MDA-modified catalase
Choi et al (78)	2018	27 35	25	inactive GO GD active GO GD (both variable functional status)	oxidants: 8OH-dG (tears), MDA	increased OS markers in tear secretions from GD patients
Diana et al (80)	2018	54 19 5	42	Untreated hyperthyroid GD Euthyroid treated GD Hyperthyroid MNTG	oxidants: 4-HNE, NOX2	OS parameters higher in untreated GD and detectable in urine samples

Abbreviations: 131I: 131 iodine; FT4: free thyroxine; GD: Graves' disease; GO: Graves' ophthalmopathy; MMI: methimazole; MNTG: multinodular toxic goiter; OS: oxidative stress; T3: triiodothyronine.

OS indexes: OSI: oxidative stress index; TAC: total antioxidant capacity; TAS: total antioxidant status; TOS: total oxidative stress; TOS*: total oxidant status; TRAP: total reactive antioxidant potential.

OS biomarkers: 4-HNE: 4-hydroxynonenal; 8-OHdG: 8-hydroxy-2′-deoxyquanosine; Antiox-cap: non-enzymatic antioxidants; ARE: arylesterase; ARS: enzymatic antioxidants; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: Glutathione; LOOH: lipid hydroperoxide; MDA: malondialdehyde; NOX2: Nicotinamide adenine dinucleotide phosphate oxidase, isoform 2; oLAb: anti-oxidized low-density lipoprotein (LDL) antibodies; ox-LDL: oxidized-low density lipoprotein; PC: protein carbonyl; PON1: paraoxonase 1; SH: thiol groups; SOD: superoxide dismutase.